EPIDERMAL GROWTH FACTOR: EFFECT UPON INTRACELLULAR ACCUMULATION OF POLYAMINES AND GROWTH

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Dedication

I would like to dedicate this thesis to : my family, who had the wisdom to discourage me from attending medical school but the foresight to anticipate my obduracy and the generosity to support it without reservation ; Shana , whose patience, love and support have been tested this past year and found to be without limit ; and Al, who has made this thesis what it is - a testament to the mischievous but inspired perseverance within us all.

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LIST OF ABBREVIATIONS

AIB - α amino isobutyric acid

DOG - deoxyglucose

DNA - deoxyribonucleic acid

DNP - dinitrophenol

EGF - epidermal growth factor

FITC - fluorescein isothiocyanate

FCS - fetal calf serum

HMW - high molecular weight

K_D - dissociation constant

K_m - substrate concentration at which velocity is half-maximal

LMW - low molecular weight

MCT - methylcholanthrene

MW - molecular weight

NGF - nerve growth factor

O. D. - optical density (absorbancy)

ODC - ornithine decarboxylase

PBS - phosphate buffered saline

RIA - radioimmunoassay

RPM - revolutions per minute

TCA - trichloroacetic acid

INTRODUCTION

In 1951 it was discovered that certain mouse tumors produced a substance that can cause remarkable proliferation of sympathetic ganglion cells (1). This nerve growth factor (NGF) was soon found to exist in significant quantities in the submandibular glands of male mice (2). Stanley Cohen found that NGF injected into newborn mice accelerated eruption of teeth and the opening of eyelids and produced an increase in the thickness of the epidermis and epithelial lining of the gastrointestinal tract. Cohen discovered that these non-neural effects were due to an "epidermal growth factor" (EGF) that was distinct from NGF yet present in the material he had originally taken to be pure NGF.

Chemical Characteristics of EGF

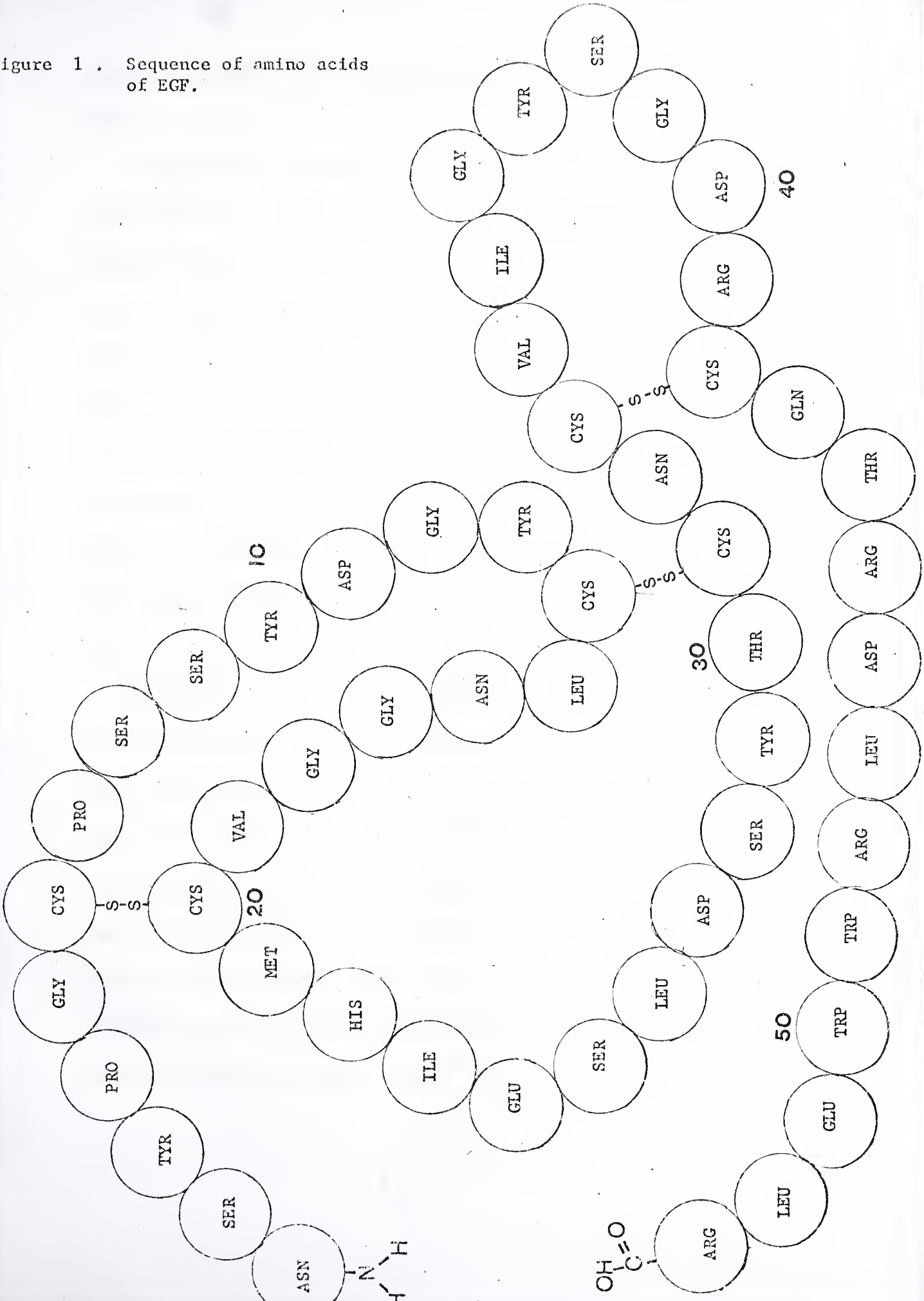
EGF was isolated and characterized and found to represent approximately 0.5% of the dry weight of submaxillary gland protein (3). The isolated material appeared pure and homogeneous by several criteria. It sedimented as a single band on the ultracentrifuge and it migrated as a single band in polyacrylamide gels. It was found to be both heat stable and non-dialyzable, and its biological activity was destroyed by pH manipulation, chymotrypsin, bacterial protease and partially by trypsin. This sensitivity indicated that EGF was a protein, and the finding of only one amino terminal residue suggested that it was a single chain polypeptide.

Amino acid analysis revealed the absence of only lysine, alanine and phenylalanine. The presence of 6 half cystine residues, and the absence of free sulfhydral groups, indicated the existence of three disulfide bridges. Low speed sedimentation equilibrium studies indicated a molecular weight of 6400 and the sequence of amino acids is shown in Figure 1.

In crude homogenates of submaxillary glands of adult male mice, EGF was found to be a component of a highly molecular weight complex (HMW EGF, MW 74,000), reversibly dissociated under a variety of conditions (e.g., pH > 8 or < 5) into a molecule of low molecular weight EGF (MW 6,400) and two molecules of EGF binding protein (MW 30,000). This binding protein was identified as an arginine esterase. The further observation that the low molecular weight EGF possessed a carboxyl terminal arginine residue suggested that it might be formed from a precursor protein by the proteolytic action of the EGF binding esterase in a fashion similar to the generation of active insulin and bradykinin.

EGF was found in significant yields only in adult male mice. This was consistent with the following observations: EGF is present only in specific tubular cells of the submaxillary gland; the tubular cells in these glands exhibit sexual dimorphism in rodents; low concentrations of EGF are found in castrated male mice and high levels in testosterone treated females (4).

Figure 1 . Sequence of amino acids of EGF.



Natural Occurrence of EGF and Biological Control of its Concentration in vivo

A solid phase radioimmunoassay (RIA) for EGF was developed in Cohen's laboratory (5, 6). This technique utilized rabbit antibody (anti-EGF) absorbed onto the walls of nylon vessels. ^{125}-I labelled EGF was displaced by as little as 10 pg of unlabelled EGF; moreover, the assay was selective as well as sensitive, and no cross reactivity was found with a variety of mouse, rat and human peptide hormones. The highest concentrations of EGF in submaxillary tissue (1200 ng/mg wet tissue) and serum (700 pg/ml) were found in mature (2 month old) male mice or mature females treated with testosterone for two weeks. These data correlated well with previous bioassay results. The solid phase assay permitted rapid and sensitive detection EGF.

Employing this technique, Cohen was able to determine that EGF receptors exhibited a K_D (dissociation constant) consistent with the serum concentrations of hormones and the plasma levels of EGF measured in mice. Later Cohen detected appreciable levels of immunoreactive EGF in a variety of tissues and body fluids (7). The following levels of the factor were found in adult mice; 10^3 ng/mg wet submaxillary tissue, 1 ng/ml plasma, 300 ng/ml milk (a level sufficient to be a factor in eyelid opening when suckling mice absorb EGF intestinally) and 10^3 ng/ml saliva and urine.

Administration of epinephrine to adult male mice results in degranulation of submaxillary gland peritubular cells (8). Therefore, it might be expected that an adrenergic stimulus could evoke the release of EGF into the bloodstream. In fact, Cohen found that the administration of 100 ug I. M. of aqueous epinephrine raised plasma levels of the polypeptide from 1 to 150 ng/ml within 1 hour and diminished submaxillary gland immunoreactive EGF content by some 50% (7).

Starkey, Cohen and Orth next produced evidence that the hormone existed in man. While EGF had not previously been isolated from species other than the mouse, murine EGF had been shown to bind to receptors and stimulate DNA and RNA synthesis in cultured human fibroblasts (9, 10, 11, 12). Since adult mouse urine contained significantly higher levels of the protein than serum, they examined human urine for the presence of EGF. Employing their previously developed RIA technique they were able to detect in human urine a material biologically and immunologically similar to mouse EGF. The amino acid composition and molecular weight were similar but not identical to murine EGF, and, predictably, human EGF was found to react one third as avidly with anti- mouse EGF antibodies. Immunoreactive EGF has also been detected in the sera of pregnant humans in concentrations as high as 6 ng/ml (13).

Taylor and Cohen had discovered that mouse EGF existed in the submaxillary gland in complex with an arginine esterase (14).

Although the function of this enzyme was unknown, the observation that other growth factors in the submaxillary gland are found associated with arginine esterases suggested to Lembach a potential role in the growth process for these enzymes (15). Since EGF was reported to be mitogenic for cultured human fibroblasts (12), Lembach exposed these cells to both high and low molecular weight EGF. He found that the growth response induced by EGF was enhanced by the presence of arginine esterase, which alone elicited no growth response. Lembach also demonstrated that the growth response induced by EGF had a low serum requirement that could not be replaced by comparable levels of bovine serum albumin or dialyzed serum (12). Lembach, however, observed that this requirement for serum could be replaced in part by low ascorbic acid supplementation (5 ug/ml) of the medium. This vitamin was found to have no intrinsic stimulatory activity, yet it stimulated the secretion of hydroxyproline into the extracellular medium 40-fold. Lembach concluded that the synergistic effect of ascorbate upon EGF proliferative activity may be mediated by the hydroxylation of collagen. In quiescent fibroblast cultures in serum-free medium, the addition of HMW EGF along with ascorbate was found to stimulate cell proliferation to a degree equivalent to a serum shift-up from 0 to 10%. Lembach concluded that EGF might require a collagenous extracellular matrix for growth activity. Such a matrix could, in turn, be either cell-derived (stimulated by ascorbic acid) or provided by serum.

Biological Action of EGF

The first observations regarding the biology of EGF were morphological changes in newborn mice. Precocious eruption of incisor teeth (as early as 6 days instead of the usual 9 days) and marked stunting of the animal's growth and hair development were all noted (3). Cohen also found that EGF could inhibit gains in body weight and size of newborn mice but it had no effect on weight gain or size when first administered 12 days or more following birth.

Cohen soon reported histological evidence that showed the observed separation of the eyelids was a consequence of the more generalized biological effect, the enhancement of epidermal keratinization and the thickening of the epidermis all over the body (16). Histological sections of the cornea of newborn mice receiving 1 ug EGF/gm body weight subcutaneously for 8 days exhibited advanced keratinization in the epidermis connecting the eyelids, while in control animals the epidermis connecting the eyelids had only begun to keratinize. These changes were not limited to the eyelid area. The back skin was increased in thickness in epidermal and keratin layers of EGF injected newborns. Administration of EGF to mice older than 12 days for a period of several weeks produced no effect on corneal or back skin keratinization. Cohen did note, though, a dramatic increase in the diameter of the experimental animals' tails and a three fold increase in the epidermal layer of tail skin.

Cohen also obtained biochemical evidence to corroborate the above morphological findings (17). The subcutaneous injection of minute amounts (2 ug EGF/gm body weight) into newborn mice produced not only epidermal hyperplasia but a 33% increase in protein and nucleic acid content per unit area of skin. The activity of several epidermal enzymes (acid phosphatase and lactic dehydrogenase, among others) rose 76% and 44% respectively when expressed per unit area of epidermis.

Effect of EGF on Cell Proliferation

EGF has been reported to stimulate the proliferation of a variety of cells. EGF was shown to stimulate the proliferation of cultivated mouse mammary cells (18), cultivated mouse mammary adenocarcinomas (19), cultivated human foreskin fibroblasts (10), cultivated human skin fibroblast (15) and 3T3 cells (21, 20). In other, less well validated reports, EGF stimulated the incorporation of ^{3}H-thymidine into DNA or a thickening of the epidermis. Cohen demonstrated by autoradiography that EGF stimulated the incorporation of ^{3}H-thymidine into the basal cells of embryonic chick epidermis (21). Bertsch and Marks (22, 23) found that EGF stimulated ^{3}H-thymidine incorporation into cultivated chick embryo epidermal cells twenty fold within 24 hours. However, the presence of desmosomes was not demonstrated, and therefore the cells were not proven to be epidermoid in origin. Maximal

stimulation of ^{3}H-thymidine incorporation occurred in the presence of serum and EGF at concentrations identical to those found in mouse plasma (7). Rabbit lens epithelial cells (24) and human skin fibroblasts (15) in culture showed similar responses to EGF. Hollenberg and Cuatrecasas found that EGF, like insulin, stimulates ^{3}H-thymidine incorporation into cultured human foreskin fibroblasts at 10^{-10} to 10^{-11}M (10). Finally, Cohen observed in histologic sections that EGF promoted the thickening of cultivated chick embryonic corneal epithelium and cultivated human embryonic skin (25).

The stimulation of ^{3}H-thymidine incorporation is consistent with a stimulation of cell proliferation. Turkington (18) reported an increase in DNA polymerase levels and an increase in the rate of cell entry into the S phase in cultivated mouse mammary cells exposed to EGF. However, it should be noted that these studies of ^{3}H-thymidine incorporation into DNA were not controlled by experiments to determine the effect of EGF on the rate of uptake of thymidine into the cells were performed. Thus, the increased incorporation of labelled thymidine into DNA could reflect changes in the intracellular pool of thymidine rather than increased DNA synthesis or increased rate of entry into the S phase.

EGF has been reported in some cases to increase the number of cell divisions (i. e., lifespan) rather than the rate of cell division. In the presence of EGF, the culture lifetime of keratinocytes grown on a matrix of x-irradiated, non-dividing fibroblasts

increased from 50 to 150 generations. EGF apparently delayed the senescence of cells by increasing the distance of the multiplying keratinocytes from obligatory terminal differentiation. EGF also enhanced the ability of the cells to survive subculture and initiate new colonies without necessarily increasing the growth rate (26).

Effect of EGF on Transport

The transport of certain compounds into cultivated chick embryo epidermis is stimulated within 15 minutes of the addition EGF. Alpha amino isobutyric acid (AIB) and uridine enter cultivated chick embryo epidermal cells at a rate two times that of untreated cells. This uptake was unaffected by cycloheximide, an inhibitor of protein synthesis, which suggests that the changes in the transport mechanism induced by EGF do not require the synthesis of new protein (27, 28).

Effect of EGF on RNA

EGF has been reported to have a variety of effects on RNA. EGF stimulated the incorporation of labelled precursors into protein and RNA fractions of cultured chick embryonic skin (29). EGF did not enhance ^{3}H-thymidine incorporation under these conditions. This indicates that DNA synthesis had not been stimulated. Puromycin (an inhibitor of protein synthesis) effectively inhibited the incorporation of ^{14}C-uridine into RNA

but actinomycin-D (an inhibitor of transcription) did not inhibit the incorporation of ^{14}C-lysine into protein. Frati et al (30) found that EGF stimulated ^{3}H-uridine incorporation into RNA in rat corneal epithelial cells. However, no one has examined the effect of EGF on the uptake of precursors as opposed to actual synthesis.

In later experiments, Cohen reported that EGF stimulated synthesis of new ribosomal subunits, increased the protein synthesizing activity of isolated ribosomes, and stimulated polysome formation in chick embryo epidermis (27). In the presence of EGF, pre-existing ribosomal monomers were converted to functional polysomes within 30 minutes. As this effect was observable even in the presence of cycloheximide, it seems that initial transfer of monosomes to polysomes does not require continual protein synthesis.

Effect of EGF on ODC

The discovery that growth hormone led to the induction of ornithine decarboxylase activity (ODC) activity in the liver (31), coupled with the similarities observed between the responses of epidermis to EGF and sundry other cell types to hormonal growth stimulation, led Cohen to investigate the relationship between ODC activity and EGF stimulation (32). He found an increase in ODC activity in cultured chick embryo epidermis that lasted less

than 6 hours. Since inhibitors of protein synthesis (cycloheximide, puromycin and fluorophenylalanine) prevented the induction of this enzyme, the rise in enzymatic activity was attributable to increased synthesis. The EGF-induced ODC activity in vivo was reflected in a 20 fold increase in putrescine accumulation within the skin of neonatal mice (32). Since Cohen found that inhibitors of protein synthesis did not affect the stimulation of other metabolic events (RNA synthesis, AIB uptake, polysome formation), the induction of ODC activity was presumed to be an early but not a primary consequence of the action of EGF.

Effect of EGF on Cell Migration

Cohen observed that one of the initial morphological effects of EGF was an apparent stimulation of cell migration (33). Aggregates of chick embryo epidermal cells were cultivated in collagen-coated culture dishes for 18 hours. Control cells consistently showed compact, flattened, colony-like areas. The presence of as little as 0.02 ug EGF/ml in the medium ,though, resulted in the spreading of cells into a loosely arranged fibroblast-like network. This morphological difference, however, may have simply been a change in the cells cytoskeleton (i.e. microtubules) or adhesive properties rather than a change in motility.

Effect of EGF on Wound Healing

Cohen examined histologically the effect of EGF on normal and regenerating epithelium in the adult rabbit. Purified protein was applied topically for 7 days without significant effect on the growth of corneal epithelium in normal rabbit eyes. In contrast, EGF stimulated the regeneration process after the excision of epithelia from rabbit cornea. Six days after the creation of the wound, control cornea had returned to the normal 4-6 layer thickness whereas the corneal epithelium of EGF-treated rabbits had 10-15 cell layers (25). The blood vessels in the limbal area were much more prominent in the experimental animals as compared to the controls. With the continued presence of EGF, this distinction disappeared and the corneal epithelium returned to its normal 6 layer thickness within 1-2 weeks after the initial wound.

Despite the initial hyperplasia, EGF did not cause an acceleration in wound closure time. When the wounded cornea were examined after 3 days, both the experimental and control groups had a monolayer of epithelial cells covering most of the central area of the cornea. The epithelium adjacent to the limbal area of EGF treated eyes, however, was 6-8 cell layers thick rather than the 2-3 layers in controls. Fluorescein staining demonstrated that complete epithelialization occurred in 4-5 days in both control and experimental animals. It would appear, then, that EGF resulted in hyperplasia rather than acceleration of wound closure.

Cohen and Ho (34) produced evidence that contradicted this data. The progressive decrease in wound size was studied with serial fluorescent photography in an in vivo investigation of standardized wounds. Corneal epithelium of rabbits was denuded by abrasion to form wounds 7 mm in diameter. It was found that exposure of the wound to concentrations of EGF between 0.05 and 2.0 mg EGF/ml saline effected a rate of epithelialization 150% that of controls. Topical steroids (0.1% Decadron) decreased the epithelial healing rate to 50% of the controls, and EGF applied simultaneously with corticosteroids exhibited no capacity to abrogate their adverse effect (35).

The ability of EGF to alter corneal healing rates was not affected over a 40-fold range of EGF concentration. Although the negative effect of steroid application suggested that inflammation might be a component of the healing process, no sign of inflammation was detected either clinically or histologically following the topical application of EGF at concentrations of as high as 2.0 mg/ml.

Effects of EGF on Tissues other than Epidermis

Jones (36) found that EGF affected a variety of tissues other than epidermis. He observed that murine EGF had a striking effect on the proliferation of cultured cells from adult rat ureter, ductus deferens, trachea, uterus, vagina, prostate and lachrymal glands.

Some tissues, such as thymus, thyroid and liver, failed to exhibit any response, even after 48 hours. EGF had a greater effect on cultures of uterine and ductucs deferens tissue obtained from younger animals. In view of the effect of EGF on tooth eruption, eyelid opening and chick embryo epithelialization (7), Jones reasoned that the most significant effect of the factor in vivo may be on embryonic organs.

These studies had shown EGF to be neither tissue nor species specific (i.e. mouse EGF had stimulated cultivated rat cells). Rechristening EGF "epithelial" growth factor (although Cohen's original name has persisted in the literature), investigators went on to reveal salient aspects of the biology of this polypeptide in other cell systems.

Effect of EGF on Neoplastic Tissues

EGF was found to stimulate the proliferation of neoplastic as well as normal cells. Turkington demonstrated that EGF stimulated the growth of explants from normal mouse mammary tissue (18) and small samples of spontaneous mouse mammary adenocarcinomas (19). Neoplastic tissues exhibited a more sensitive response to the growth factor than their non-neoplastic analogues, with a maximal proliferative effect achieved at concentrations of 8×10^{-11} M EGF. EGF--treated cultures evinced an increase in DNA synthesis as compared to controls, reflecting primarily an increase in the number

of cells engaged in DNA synthesis. A parallel increase in the mitotic index proved that an actual increase in cell proliferation was taking place. As was the case with normal mouse mammary tissue, EGF stimulated an increase in the mitotic index and the rate at which cells entered the S phase but had no effect on the length of the S phase.

The effects of EGF were not limited to established neoplasms, and Reynolds and Cohen (37) found it to have a synergistic effect upon the carcinogenicity of a polycyclic hydrocarbon, 3-methylcholanthrene (MCT). It had been observed that EGF promoted pronounced atypical hyperplasia and increased mitotic activity in the burned skin of mice in vivo (37). (Unfortunately, Reynolds did not describe precisely the extent or depth of the burns. It would have been informative to have had data on EGF's effects on proliferative activity and reepithelialization in skin that had been substantially deprived of dermis and dermal adnexa.) Observation of this atypical hyperplasia led to Reynolds' collaborations with Cohen to investigate the interaction of EGF and MCT. When purified EGF was injected daily subcutaneously beneath topically applied MCT, carcinomas appeared sooner and with more frequency than with the control group receiving topical MCT without EGF injections.

Some tumors, however, were not influenced by EGF. Waghe investigated the apparent "non-species specificity" as applied to human tissues, both normal and neoplastic (38). In addition to normal kidney and breast, a variety of tumors were employed - breast carcinoma, Wilms and neuroblastoma. Proliferation in all these cultivated tissues, however, were found to be uninfluenced by murine EGF or its antiserum. Similarly, FITC (fluorescein isothiocyanate) labelled antisera to the purified protein failed to stain any cellular components of the human tumors or normal tissue.

Binding of EGF to Tissues and Cultivated Cells

Studies to date attempting to localize EGF receptors have utilized EGF labelled with ^{125}I or ^{131}I. One group (39) injected ^{131}I-labelled EGF into mice and sacrificed them at 30 minute intervals. At each interval, various organs were isolated and the amount of radioactivity in each was determined. They found that only two tissues concentrate labelled EGF with respect to blood - the epidermis and epithelial cells of the cornea. These results are not consistent with other data indicating that cultivated fibroblasts bind EGF and respond characteristically to it (10). These same workers (30) later reported that ^{131}I-labelled EGF bound specifically to rat corneal epithelium; however, their method of isolation of the corneal epithelium by scraping did not insure the removal of all fibroblasts from the underlying tissue.

Hollenberg and Cuatrecasas studied the binding of ^{125}I-labelled EGF with cultivated skin fibroblasts (10). The binding of this labelled protein was found to be saturable and specific, and a synergistic effect was observed with 5 and 10% fetal calf serum. They determined an apparent dissociation constant of 3×10^{10} liters/mole and an average number of binding sites to be 4×10^{4} per cell. EGF did not exhibit any significant binding affinity for those cell types (PB and WL-38 fibroblasts) that were only minimally responsive to the hormone. Cholera toxin in very low concentrations (2×10^{-14} M) was found to inhibit totally the stimulatory effect of EGF without affecting its binding, indicating that EGF and cholera toxin have different receptors at the cell surface. As the effects of cholera toxin are presumably mediated by the elevation of intracellular levels of cyclic AMP (cAMP) (40), Hollenberg and Cuatrecasas hypothesized that EGF might stimulate DNA synthesis by decreasing cAMP levels.

Cohen next prepared an ^{125}I-labelled EGF and confirmed the biological effect of the labelled hormone (11). Competitive inhibition tests demonstrated specificity of binding, which was confirmed by the observation that the inclusion of 10% rabbit anti-EGF serum inhibited the binding of labelled EGF by greater than 90%. Iodination of EGF, therefore, did not appear to alter the binding affinity of the molecule or its capacity to stimulate fibroblast proliferation. Cohen

also obtained values for a binding constant and number of binding sites within the same order of magnitude as those found by Hollenberg and Cuatrecasas (4.3×10^{10} liters/mole and 1×10^{5}/cell versus 3×10^{10} liters/mole and 4×10^{4}/cell).

Cohen also examined the capacity of a variety of cell lines to bind labelled EGF, and the following cells were shown to exhibit specific binding - human foreskin fibroblast, mouse embryo fibroblast (3T3), human melanoma (EPO-H8), mouse mammary carcinoma (Ae-963) and chick embryo epidermis. Hollenberg and Cuatrecasas later demonstrated the presence of trypsin-sensitive EGF binding sites in liver and placental membranes (41).

The fate of the ^{125}I-labelled EGF bound to cultivated fibroblasts was also determined. Maximal binding at 37^{o}C was observed in 40 minutes and Cohen noted that prolonged incubation in the presence of unlabelled EGF resulted in a loss of cell bound radioactivity. After maximal binding had been reached, cell bound radioactivity decreased with a half life of 1 hour. This observation prompted investigation into the metabolic fate of the cell bound ^{125}I-EGF (12) and it was shown that the time dependent decrease in bound radioactivity was not due to inactivation of the ^{125}I-EGF remaining in the medium. Indeed, this material was found to be fully active 6 hours after addition to the cultures. Saturation of binding was achieved after 5 hours of incubation. The radioactive

material released from the cells was determined to be ^{125}-I monoiodotyrosine. The addition of excess quantities of labelled EGF did not increase the amount of free ^{125}I-tyrosine. In addition, media incubated with fibroblasts was not capable of degrading ^{125}I-labelled EGF. Presumably, degradation was taking place on the surface or within the cell itself, but these studies did not differentiate between proteolysis occurring at the surface or within the cell following endocytosis of ^{125}-I EGF. The inhibition of release of cell bound radioactivity by the inhibitor of protease activity, tosyl-lysine CH_2Cl,and benzyl guanidobenzoate furthermore suggested that one of the components of the degradation system might have trypsin-like properties.

Although Cohen had shown that the release of cell bound radioactivity ultimately brought about an increase in (^{125}-I) monoiodotyrosine in the medium, it is plausible that this represented a limited proteolysis of a single iodinated tyrosine residue rather than the degradation of an entire EGF molecule. Such a scheme might also explain the observation that cell bound radioactivity continued to be released several hours after cell binding of labelled EGF ceased.

Primary Site of Action of EGF

Cohen attempted to determine whether EGF acted directly on the epidermis or whether the epidermal effects were the consequence

of an initial effect on dermis. Using collagenase, he separated epidermal sheets from the underlying dermis in chick embryo skin and exposed the epidermis to EGF at concentrations of 5 ug/ml in two different environments. In the first set of experiments, the sheets were layered onto the surface of "killed" (frozen and thawed four times in succession) dermis, and for the second set of studies, the sheets were simply laid on a millipore filter. Previous work indicated that sheets of chick embryo epidermis isolated and cultured in this manner can survive and keratinize on a substratum of frozen-killed dermis or collagen (42) but fail to proliferate or retain columnar orientation of basal cells (42, 43). Cohen found that the addition of EGF could restore the orientation and proliferation of basal cells. In the culture combining epidermis with frozen-thawed dermis, the effect of the hormone was thus indistinguishable from its proliferative effect on epidermis in intact skin (44). When epidermis was layered onto collagen or filter paper and incubated with EGF, proliferation was stimulated although basilar columnar orientation was lost (44).

Cohen thus demonstrated that EGF could promote epidermal proliferation and keratinization in the absence of intact, viable dermis, providing the basal cells were attached to some substratum. Cohen's methodology, however, does not exclude the possibility that fibroblasts might have an important mediating effect on EGF's

action on epidermis. Other investigators have found that EGF stimulated the proliferation of fibroblasts (9, 10, 11, 12). Also, the isolation of pure epidermal sheets with collagenase often results in contamination by dermal fibroblasts. Even if the sheets are rigorously washed, it is highly likely that portions of rete pegs remain attached to the epidermis. Thus, the observed effects of EGF on the "isolated" epidermis may have been mediated by contaminating fibroblasts.

Possible Mechanism of Action of EGF

Considering the similar response of a variety of cells to both insulin and EGF and the structural similarities between the two hormones (three disulfide bridges, a terminal asparigine residue and a molecular weight in the region of 6000 and storage in a form associated with another peptide) it is not surprising that many investigators consider the mechanisms of action of these two substances to be similar. Hollenberg and Cuatrecasas were the first to propose that EGF, much like insulin, might promote cell proliferation by first stimulating guanalyte cyclase activity (45). This was based upon the observations that (A) increased levels of cyclic AMP have been associated with reduced rates of proliferation in many cell types (46) and that (B) cholera toxin (which increases intracellular levels of cAMP) could inhibit the growth effect of both

insulin and EGF (40). It is possible that the basic action of all growth hormones may closely parallel the way in which insulin affects fat and liver cells.

In a sense, the biologic action of EGF could be described in terms of its insulin-like activity. If this were true, however, there are instances when EGF activity would exceed that of the standard (insulin), most notably in its ability to stimulate cell proliferation of mammary carcinoma in concentrations 1/100th that of insulin (19, 47). Although the time course in vitro and magnitude of stimulation are comparable for the two hormones, EGF, unlike insulin, is an effective growth stimulator in physiologic concentrations. On the other hand, purified EGF has no insulin-like activity upon isolated fat cells nor does it bind to these cells or displace insulin from its receptor (10). In addition, Turkington has shown that EGF, unlike insulin, is a poor synergist with prolactin for the induction of milk proteins in differentiated mammary epithelial cells (18).

EGF and Gastric Acid Secretion

Peptide hormones such as insulin and gastrin, in addition to their effects upon tissue growth, have a well-defined short-term action on gastric acid secretion. Bowers (48) found that histamine and EGF simultaneously administered to rats subcutaneously could reduce the total amount of gastric acid secretion induced by

histamine by up to 80%. This effect was produced by doses as low as 10 ug/kg in rats. This represents an amount of EGF far less than that necessary to achieve the early opening of eyes and eruption of teeth (48,49). Moreover, this effect appears to be specific as it produces no changes in respiration, heart rate or temperature.

EGF is found primarily in the salivary gland although it appears in appreciable quantities in saliva as well as other body fluids (7). Interestingly there is an association between human saliva and the inhibition of acid secretion in the stomach, an association first observed in 1949 (50). Ances' discovery of increased concentration of EGF in the serum of pregnant humans but not males or non-pregnant females (51) may explain the known low incidence of peptic ulceration during pregnancy. EGF is known to stimulate ornithine (32) and histidine decarboxylase (52) in skin and a variety of other tissues. Although this effect has not been studied in relation to gastric mucosa, it is known that a number of inhibitors of gastric secretion (e.g., metiamide, burimamide, antigastrin) also stimulate gastric mucosal activity (53).

Polyamines and Growth

The polyamines - spermine and spermidine - and their diamine precursor, putrescine, have been coincidentally if not causally associated with a variety of normal and pathological conditions of

increased rates of cell proliferation. A high polyamine content is found in cells in malignancies (54) and psoriasis (55), as well as developing chick embryos (56), regenerating rat liver (57), growth hormone stimulated fibroblasts (58) and lymphocytes stimulated to proliferate by mitogenic lectins (59).

In addition to their elevation in rapidly growing tissues, the polyamines have been shown to be essential growth factors for bacteria (60), certain insects (61), molds (62) and plant cells (63). Polyamines are derived from ornithine by the enzyme ornithine decarboxylase (ODC) (Figure 2). An ODC inhibitor, alpha methyl ornithine, has been shown to block the proliferation of rat hepatoma cells in culture (64). The addition of putrescine, spermine or spermidine to these cells, however, results in immediate resumption of proliferation. The addition of polyamines acts to inhibit ODC in the absence of alpha methyl ornithine (68). These findings suggest that the presence of polyamines (either synthesized endogenously or obtained from exogenous sources) acts as a direct stimulus for cell proliferation.

At present only two ways are known by which cells can increase the concentration of polyamines within the cytosol: an increase in ODC activity and the stimulation of polyamine transport from the external milieu. ODC activity is very low in static, nongrowing

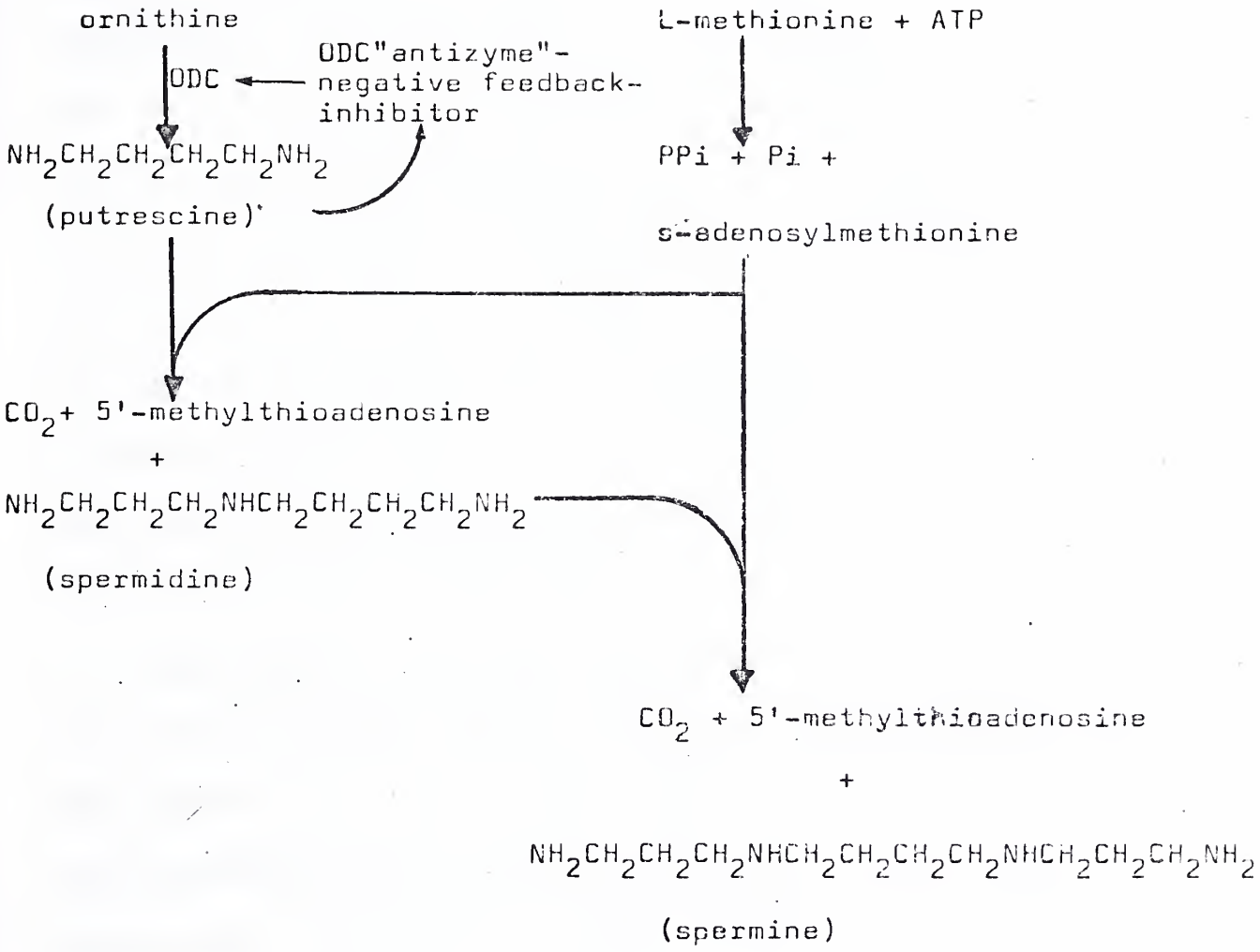


Figure 2 . Pathways of polyamine synthesis

tissues, but when proliferation is stimulated with growth hormone, (inter alia) the levels of putrescine undergo a swift and dramatic increase. If the stimulus is removed, ODC activity returns to control levels or below very rapidly, with a 1/2 life of 15 minutes (66). Recently, putrescine transport into human fibroblasts was found to be greatly increased when the cells were initiated to proliferate by the addition of serum or insulin. It would appear, then, that the intracellular accumulation of polyamines can be accomplished by transport across the cell membrane as well as by synthesis within the cytosol. These results indicate that putrescine (the product of ODC) or the polyamines (of which putrescine is the precursor) are cues for cell division.

Such signals are precisely modulated. Heller, Fong and Canellakis (67, 65) have found that putrescine induces the synthesis of a protein that combines with an inhibits ODC. This "antizyme" and ODC both have comparably short 1/2 lives. This indicates that the interplay between ODC, the polyamines, and the inhibitors derived from ODC would constitute a sensitive modulator of enzyme activity. The induction of the synthesis of a protein inhibitor to an enzyme (ODC) by its immediate product (putrescine) represents a new model for enzyme regulation. Investigations into regulation by this antizyme may lead to the precise identification of what is being regulated by the polyamines.

Effects of Polyamines on Cellular Substances Related to Cell Proliferation

Although the physiological role for putrescine and the polyamines is not completely understood, there is increasing evidence that they play a direct role in the stimulation of cell proliferation. Polyamines are known to intercalate into the DNA double helix, thereby making the molecule more flexible. This intercalation is probably the result of the electrostatic attraction between the positively charged polyamine and the negatively charged DNA. Polyamines are also known to stabilize ribosomal RNA (69) and to stimulate DNA polymerase activity (70), and there is evidence that ODC is an initiation factor for RNA polymerase I (71).

Polyamines and EGF-Stimulated Cell Proliferation

The mechanism by which EGF stimulates cell proliferation is unknown; however, there are striking similarities between the effects of EGF and the polyamines on growth and related metabolic activities. EGF injected into mice increases ODC activity and, in turn, the levels of putrescine synthesized from ornithine. Moreover, both EGF and the polyamines have been shown to stabilize RNA and promote the association of ribosomal subunits into polysomes. It was these correlations that directed our attention to the relationship between EGF, the polyamines and growth, and ultimately led

us to investigate whether the effect of EGF upon cell proliferation was mediated by polyamines.

The present investigation of the effects of EGF on the accumulation of polyamines within cultivated fibroblasts showed that EGF promoted the transport of both putrescine and spermine and stimulated ODC activity in cultivated human skin fibroblasts. These findings suggest that EGF stimulates cell proliferation by increasing intracellular levels of polyamines. Experiments were conducted to determine the factors that influence this transport system in an effort to reveal the precise biochemical mechanism of action of EGF. It was discovered that the polyamines - putrescine, spermine and spermidine - are transported by the same mechanism and that ornithine, 2-deoxyglucose, thymidine and alpha-amino isobutyric acid are not transported through this channel. A protein with a short half life selectively inhibits putrescine transport, and inhibition of the synthesis of this protein increases the maximum velocity of putrescine transport without affecting the K_m.

MATERIALS AND METHODS

Isolation and Identification of EGF-

Epidermal growth factor (EGF) was prepared according to Cohen (72). Submaxillary glands were harvested from 89 adult male Swiss Webster mice, each weighing in excess of 40 grams. The mice were killed with chloroform and the submaxillary glands excised and stored on dry ice at -50°C for several weeks. These glands, weighing 14.56 gms, were thawed at room temperature and homogenized with 58.43 ml of cold 0.05 M acetic acid in a Waring Blender (Model 700A) at 4° for three, one minute intervals. The final pH was adjusted to 4.5. The resulting crude homogenate, containing very few remaining visible remnants of the glands, was frozen overnight. This homogenate was thawed at room temperature and centrifuged at 106,500 X G (40,000 RPM, type 50 Ti rotor) for 30 minutes on a Beckman Model L3-50 Ultracentrifuge. The resulting supernatant was decanted through glass wool to remove fat. The pellet was washed with 2 ml. of 5×10^{-4} M acetic acid. The washings were centrifuged in the same manner as the homogenate. The wash was repeated once and the supernatant fractions were combined and lyophilized, and the pellet was discarded.

The dry residue was combined with 5.0 ml cold 1 N HCl in order to lower the pH rapidly. The mixture was then diluted with

12.85 ml of 0.05 N HCl and then briefly sonicated with a Sonifer Cell Disrupter (Model W185D) (Heat Systems and Ultrasonics, Inc. Plainview, N.Y.) for 5 bursts of 5 seconds duration each at 4° (Microtip setting #4). The pH of the mixture was then adjusted to 1.5 with 1 N NaOH, and then centrifuged at 100,000 X G for 30 minutes at 4°. The pellet was washed once with 2.5 ml of 0.05 N HCl, recentrifuged, and the supernatants combined. The yield of the resulting rust-colored solution was 21.0 ml (pH 1.5). This solution was chromatographed on Bio Gel P-10 (100-200 mesh) in a 95 X 2.5 cm column equilibrated with a buffer composed of 0.05 N HCl and 0.15 N NaOH at 5°. The column was packed at 170 cm of buffer pressure and operated at a flow rate of 16 ml per hour. Forty fractions of 20 ml each were collected over a period of 36 hours. The absorbancy of these fractions was measured on a Gilford Spectrophotometer at 280 nm (see Figure 3). Fractions 26-35 (approximately 200 ml) were pooled and adjusted to pH 5.5 with 1 N NaOH and concentrated to 12 ml by pressure ultrafiltration using a UM-2 membrane (rated to withhold particles larger than 1000 Daltons) at 65 psi of N_2. This preparation was then applied to a DEAE-52 cellulose column (1.5 X 20 cm, equilibrated with 0.02M ammonium acetate, pH 5.6 at 5°) and packed at 50 cm buffer pressure and run at 29 cm buffer pressure. This solution was

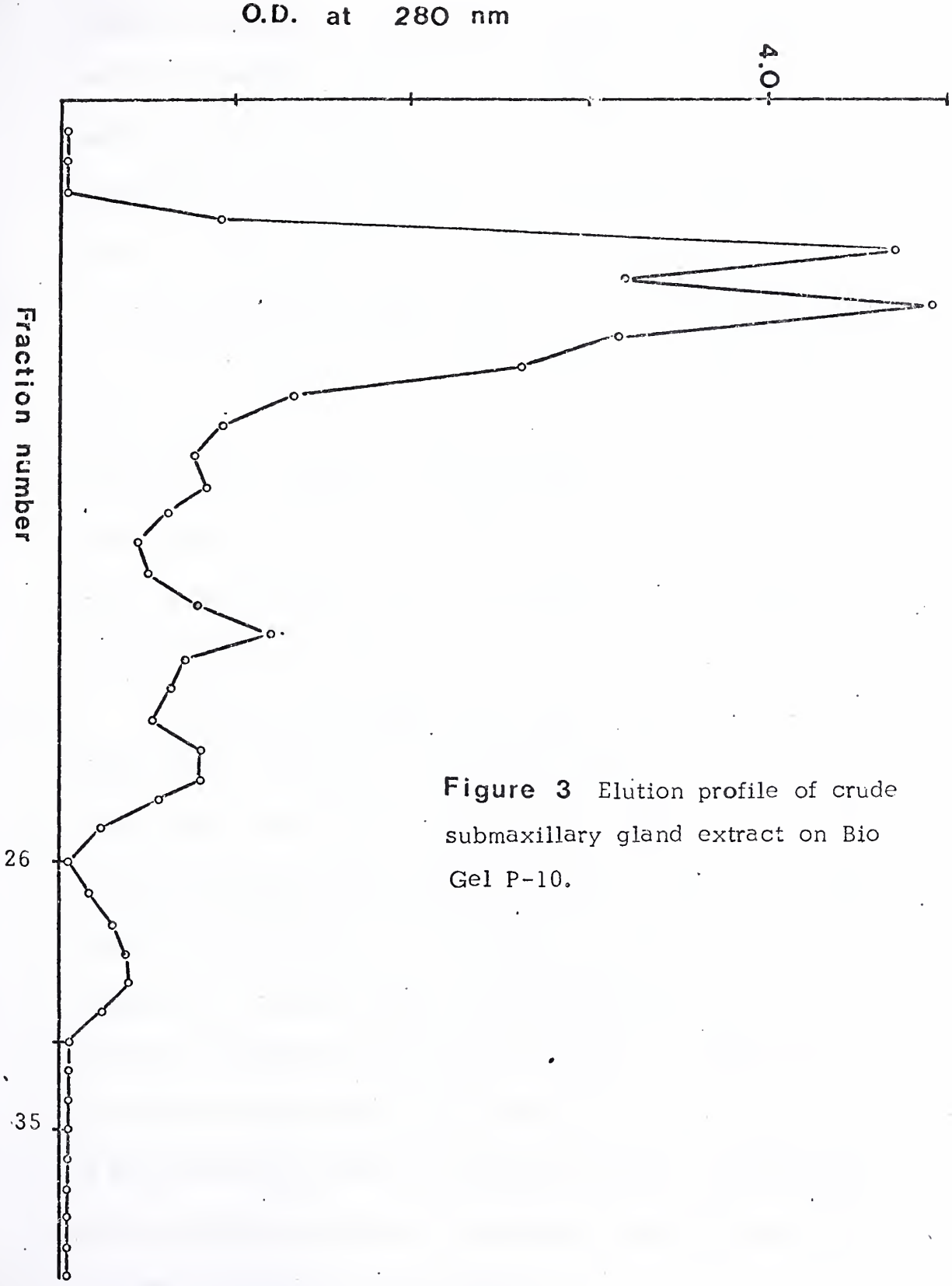


Figure 3 Elution profile of crude submaxillary gland extract on Bio Gel P-10.

applied to the column. The column was then washed with 60 ml of 0.02M ammonium acetate prior to starting the 0.02 to 0.2M ammonium acetate gradient in order to improve resolution. (This gradient was formed by allowing 0.2 ammonium acetate buffer (pH 5.6) to flow into a 120 ml constant volume mixing chamber containing the 0.02 M buffer, also at pH 5.6). The flow rate was 22.5 ml/hr, maintained by a Buchler polystaltic pump. Each fraction was 6 ml, and the peak fractions (#30-37) were pooled (Figure 4). This solution was lyophilized overnight (in 20 ml of 0.05 N acetic acid and 5.7 ml glacial acetic acid brought to 100 ml with distilled H_2O). The weight of the final, fiberous lyophilized EGF was 35 mg.

In order to ascertain the homogeneity of the product, 50 ug of EGF was run on polyacrylamide gels according to Taylor and Cohen (73). The material migrated as a single band (Figure 5). To verify the immunologic identity of the EGF, antibodies were prepared by injecting into the footpads of a rabbit 2.2 mg EGF diluted in 0.5 ml of distilled H_2O and suspended in 0.5 ml of Freunds complete adjuvant. Four weeks later a similar booster injection was made which was followed by the harvesting of 10 ml of blood drawn from an ear vein one week later. The blood was allowed to clot overnight and 5 ml of clear, straw colored serum was drawn off from the clotted tube.

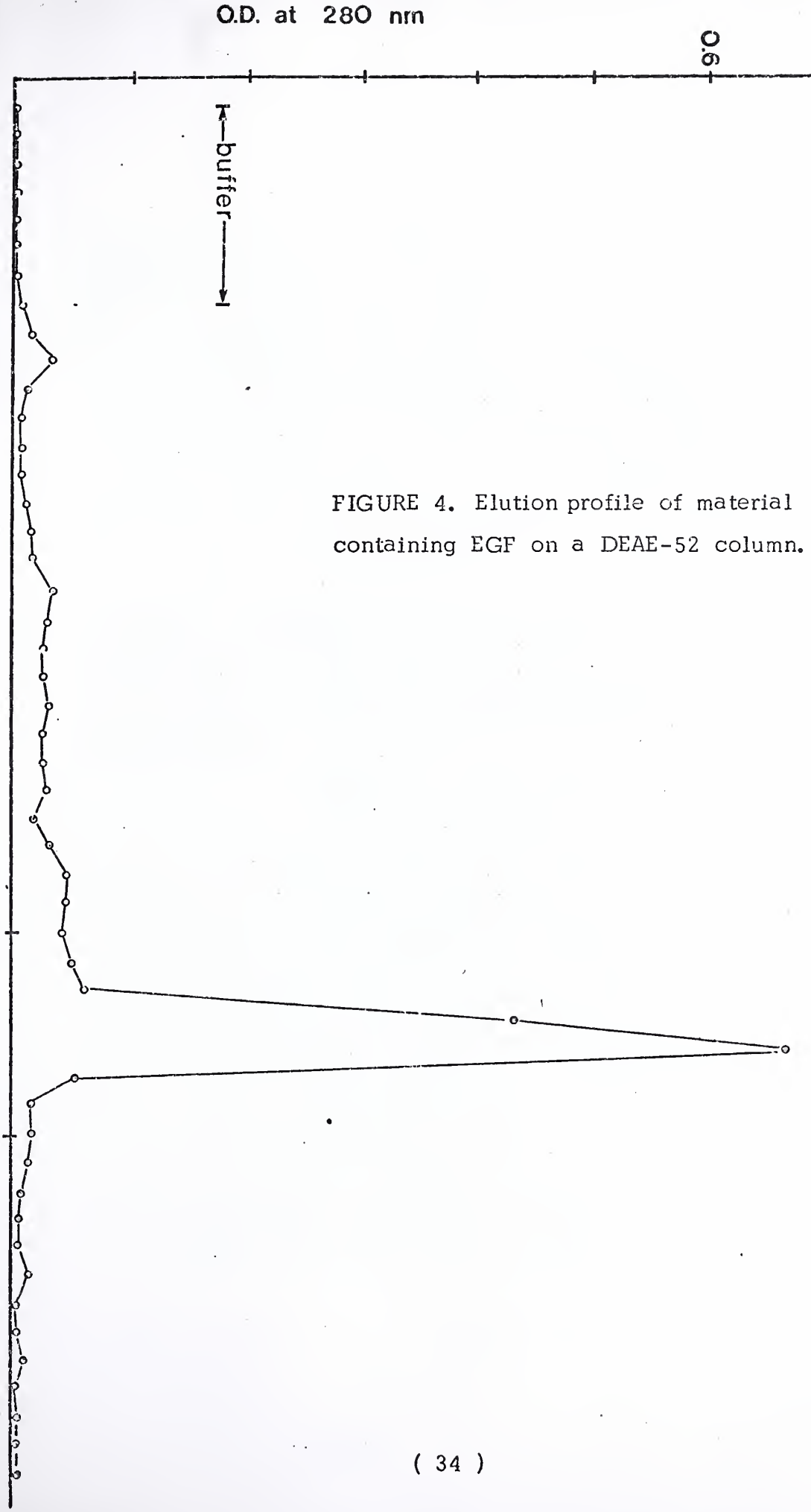


FIGURE 4. Elution profile of material containing EGF on a DEAE-52 column.

FIGURE 5. Migration of 50 ug prepared EGF on polyacrylamide gels. Gel on the left is 7.5% polyacrylamide and gel on the right is 12.5% polyacrylamide.

In Ochterlouny plates, 50 ug of EGF obtained from Dr. S. Cohen of Vanderbilt University was placed in the center well, and dilutions of 1, 1:2, 1:10, 1:100 and 1:1000 of the rabbit serum placed in the surrounding wells. The plates were incubated at 37° overnight, and washed with 0.9% NaCl. The plates were soaked overnight with 0.9% NaCl to remove free protein and then stained with 0.5% Coomassie blue for 4 hours at room temperature. The plates were then destained in a mixture of methanol, acetic acid and water (1: 1:2). The single bands, clearly seen surrounding the wells containing full and half strength serum (Figure 6) indicate that the antibodies were specific for EGF.

EGF thus prepared was also shown to be biologically active as well as immunologically and chemically identical to the Cohen EGF when injection into newborn mice caused stunting of growth (Figure 7) and early eye opening and dental eruption as has been described by Cohen (8).

2 ug of EGF in .025 ml ul of H_2O was injected into 2 day old newborn mice each day for 8 days. 8 days following birth the eyes had opened in the five EGF treated animals while eye opening in the 5 control animals did not occur for an additional 7 days. Earlier dental eruption was also observed in the experimental mice. Figure 7 demonstrates the stunting of growth observed in the EGF

FIGURE 6 . Reaction of EGF and rabbit antibodies to prepared EGF in Octerlouny plates. 50 ug of EGF was placed in central well, and dilutions of rabbit antiserum were placed in the surrounding wells in the following clockwise pattern - 1:1, 1:2, 1:10, 1:100 and 1:1000. Antigen, antibodies and plates were prepared as described in Materials and Methods.

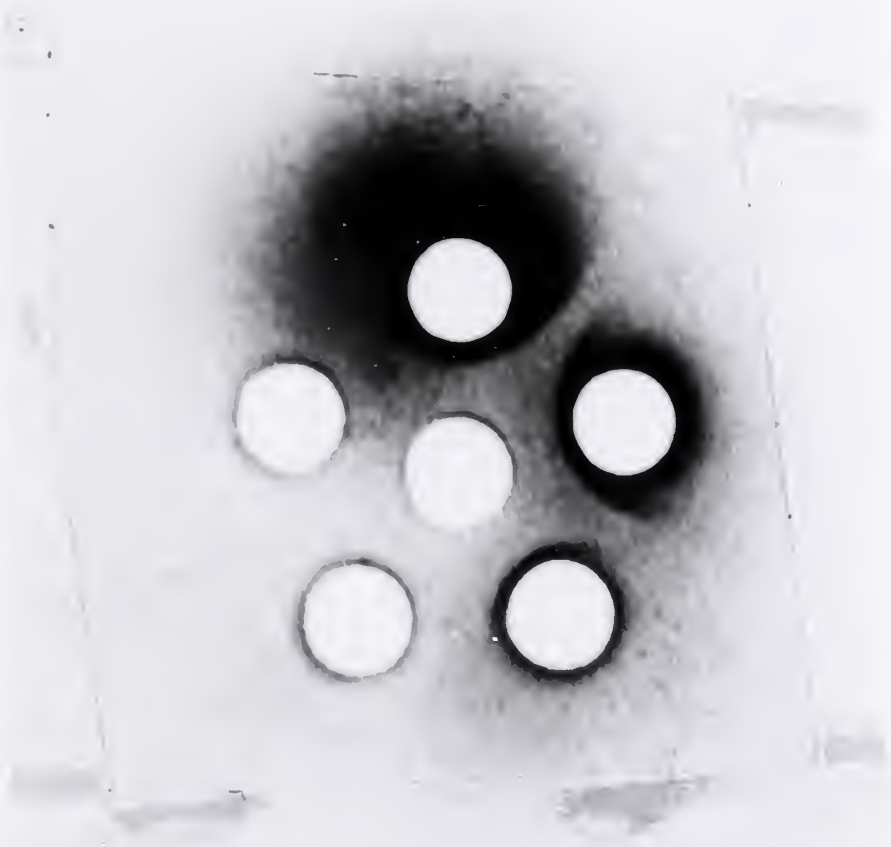


FIGURE 7 . Stunting of growth in newborn mice injected with EGF as described in Materials and Methods. The mouse on the right was treated with EGF and the control animal is on the left.


METRIC
1
2
3
4
5
6
7
8
9
10

treated mice 14 days following birth. The experimental animals were also found to be 25% lighter than the experimental mice (5.6±0.3 gm versus 7.0±0.3 gm) at this time.

Tissue Culture

Human skin fibroblasts (ATCC CRL), 1295 KB cells (CCL 19), Cloudman melanoma cells (NCTC 3960 CCL 53), and macrophages (P3 88 D1) derived from DBA mice were grown in Dulbecco's medium supplemented with 10% fetal calf serum (FCS) (Microbiological Associates) and 50 ug/ml gentamycin (Schering Corp.). Cells were incubated at 37^{o} in air containing 5% CO_2.

Measurement of Transport

Transport was measured according to the method of Pohjanpelto (74). Cells were seeded as nonconfluent cultures at 8×10^4 cells per Falcon plastic tissue culture dish (3.5 cm in diameter) in 2 ml of medium containing FCS. After 24 hours the medium was removed and replaced with 1 ml of medium without serum. After 18 hours, appropriate additions were made in 0.1 ml of medium which did not contain serum. At the indicated times, 0.5 ml of ^{3}H-putrescine in medium without serum was added at a final concentration of 1.25 uCi/ml (20.6 Ci/mmole, New England Nuclear). After 15 minutes at 37^{o}, entry of the labelled putrescine was terminated by the addition of 1 ml of 10^{-3}M unlabelled putrescine in phosphate buffered saline (PBS) (0.01 M sodium phosphate,

0.15 M NaCL, pH 7.4). The dishes were rinsed 3 times with PBS at 4° and 1 ml of 5% trichloroacetic acid (TCA) was added for 30 minutes. The soluble TCA extract was removed and mixed with scintisol (Isolab; Akron, Ohio) and the radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer. Only 2% of the labelled putrescine taken up by the cells was present in the TCA precipitate. Transport of ^{3}H-spermine (44.3 Ci/mmole), ^{3}H-alpha-amino-isobutyric acid (AIB) (9 mc/mmole), 2-deoxyglucose (2-DOG) (7.9 Ci/mmole), methyl-^{3}H-thymidine (6.7 Ci/mmole), and DL-ornithine ($I^{-14}C$) (49.9 mCi/mmole), all from New England Nuclear Corp., were determined in the same way as for ^{3}H-putrescine. Label remaining in the TCA precipitate was determined as described by Pohjanpelto (74). The final concentrations of all compounds was 10^{-7} M. Cycloheximide (Sigma Chemical Corp.) was added in 100 ul of medium without serum.

Other Analyses

Cells were counted with a Coulter Counter and Channelyzer (Coulter Electronics, Inc.) and the amount of protein per cell was determined by the method of Lowry et al (75) using bovine serum albumin as a standard. Fetal calf serum was dialyzed against PBS, pH 7.4 (three changes over a period of 2 days) and sterilized by passage through a 0.22 um Millipore filter. Descending paper chromatography was performed on Whatman no. 1 paper using a

solvent system of n-butanol-pyridine-glacial acetic acid-H_2O (4:1:1:2) (59). Ornithine decarboxylase (ODC) activity was measured according to the method of Stastny and Cohen (32) using DL-ornithine (1-^{14}C) (New England Nuclear Corp., 49.9 mCi/mmole). The amount of $^{14}CO_2$ released from the reaction mixture was linear with time for at least 50 minutes and proportional to the activity of ornithine decarboxylase. The $^{14}CO_2$ released was trapped in hyamine which was dissolved in scintillation fluid (4g PPO, 50 mg POPOP in 1 liter toluene). Radioactivity was counted in a Nuclear Scintillation Counter.

RESULTS

Table 1 shows the effects of EGF, insulin and serum on putrescine transport in several cell lines. EGF, insulin and fetal calf serum (FCS) stimulated putrescine transport in human skin fibroblasts and KB cells, while only FCS stimulated putrescine entry into melanoma cells and macrophages. The accumulation of putrescine in human skin fibroblasts was linear for at least 20 minutes (Figure 8). A 6 hour exposure to EGF (1 ug/ml) doubled the rate of accumulation (Figure 9). Paper chromatography of TCA extracts of EGF-treated and untreated skin fibroblasts exposed for 20 minutes to 10^{-7} M ^{3}H-putrescine revealed that 99% of the isotope was in putrescine. The concentration of putrescine in these cells was 50 to 150 times that of the medium, and only 15% of the accumulated label was lost into the medium when the cells were incubated in a 10^{4} fold excess of unlabelled putrescine (74). These results are consistent with active transport of putrescine. Neither BSA, ferritin nor highly purified alpha or beta MSH stimulated putrescine transport in human fibroblasts.

The K_m of putrescine transport (2.2×10^{-7} moles/liter) was unaffected by EGF (6 hour exposure). The V_{max} was doubled by EGF (1.42 vs. 0.77×10^{-3} moles putrescine/minute/50,000 cells).

cpm/15 min/8×10^4 cells

treatment/ cell type	KB		Skin Fibroblasts		Cloudman Melanoma		Macrophage	
	cpm	% of control	cpm	% of control	cpm	% of control	cpm	% of control
Control	4272±260	100	7339±288	100	5736±793	100	1334±257	100
EGF	8258±695	193	13,583±1200	185	6012±250	105	1393±103	104
Insulin	9472±459	222	30,800±50	419	7310±156	127	1295±41	89
FCS	8168±791	191	21,315±532	219	9319±111	162	2460±380	184

Table 1. Effect of a 6 hour exposure to EGF (1 μg/ml), insulin (10 μg/ml), or FCS (10%) on putrescine transport in human skin fibroblasts, KB cells, pigmented Cloudman melanoma, and macrophages.

Figure 8. Rate of accumulation of putrescine over 20 minutes in cultured human skin fibroblasts. Cells were exposed to EGF for 12 hours.

FIGURE 8

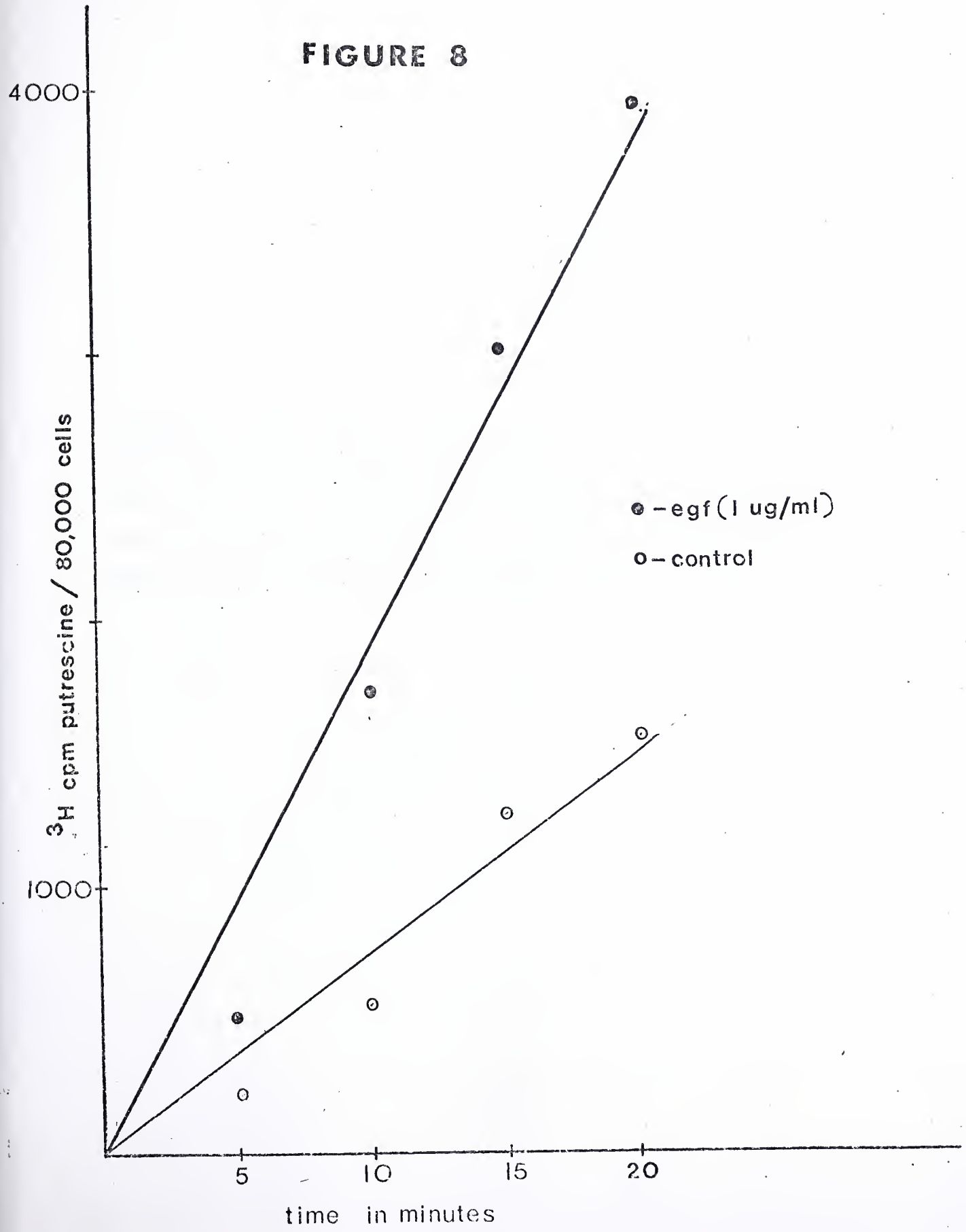
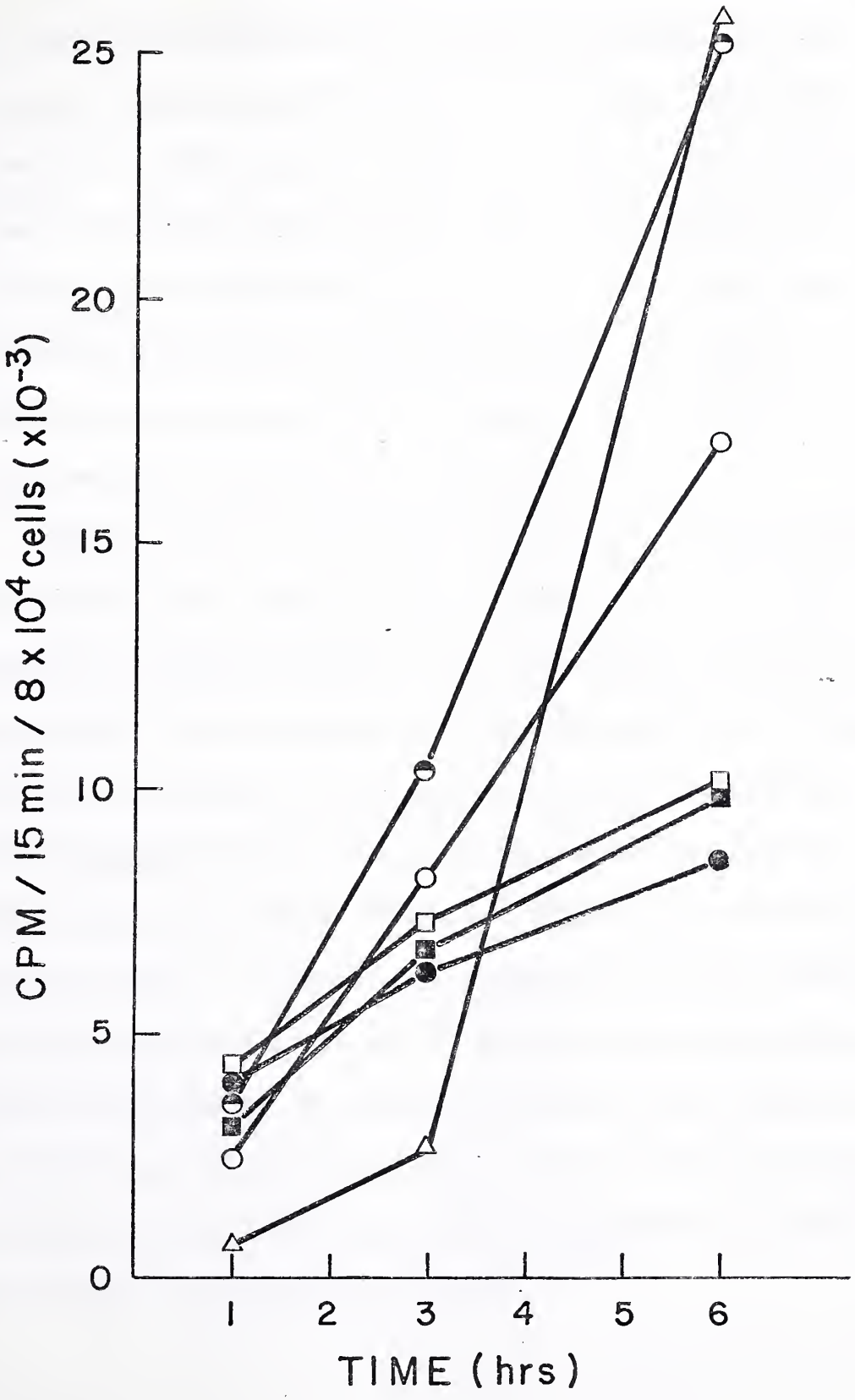

4000
1000
^3H cpm putrescine / 80,000 cells
● – egf (1 ug/ml)
o – control
5
10
15
20
time in minutes

FIGURE 9 . Time course of stimulation of ^{3}H-putrescine entry into human skin fibroblasts (●) control; (○) EGF (1 μg/ml); (◐) insulin (10 μg/ml);(△) FCS (10%); 8-bromoguanosine-3'-5'-cyclic monophosphoric acid, 10^{-6} M (▣) and 10^{-4} M (□). Measurements were made as described in text.


25
20
15
10
5
0
CPM / 15 min / 8 x 10^4 cells (x10^-3)
1
2
3
4
5
6
TIME (hrs)

The stimulation of polyamine transport by EGF was selective. EGF stimulated significantly the transport of both putrescine and spermine but did not stimulate transport of ornithine, alpha-AIB, thymidine or 2-DOG (Table 2). Spermine probably shares the same transport mechanism with putrescine, since either spermine or spermidine at concentrations equimolar to putrescine inhibit the transport of putrescine by one half. In contrast, the entry of neither putrescine nor spermine was influenced by ornithine in concentrations 10^4 greater than the polyamine (Table 3).

The time course of stimulation of putrescine transport in skin fibroblasts by EGF, insulin and FCS is shown in Figure 9. The stimulation is apparent between 3 and 6 hours after the addition of these agents. Initial inhibition of putrescine transport was observed with FCS. As dialyzed FCS also produced this initial inhibition, this effect is probably not due to the presence of putrescine in FCS. Monobutyryl cyclic GMP (10^{-4}M and 10^{-6}M) stimulated putrescine transport slightly. Dibutyryl cyclic AMP (10^{-3}M) inhibited putrescine transport by 50% (4.4 vs. 2.1×10^{-10} mmole/10 min/80,000 cells) and reduced the stimulation of putrescine transport by EGF 50% (8.9 vs. 4.0×10^{-10} mmole/10 min/80,000 cells). EGF and insulin stimulated putrescine transport half-maximally at concentrations of 1.6×10^{-9}M and 1.74×10^{-9}M respectively (Figure 10).

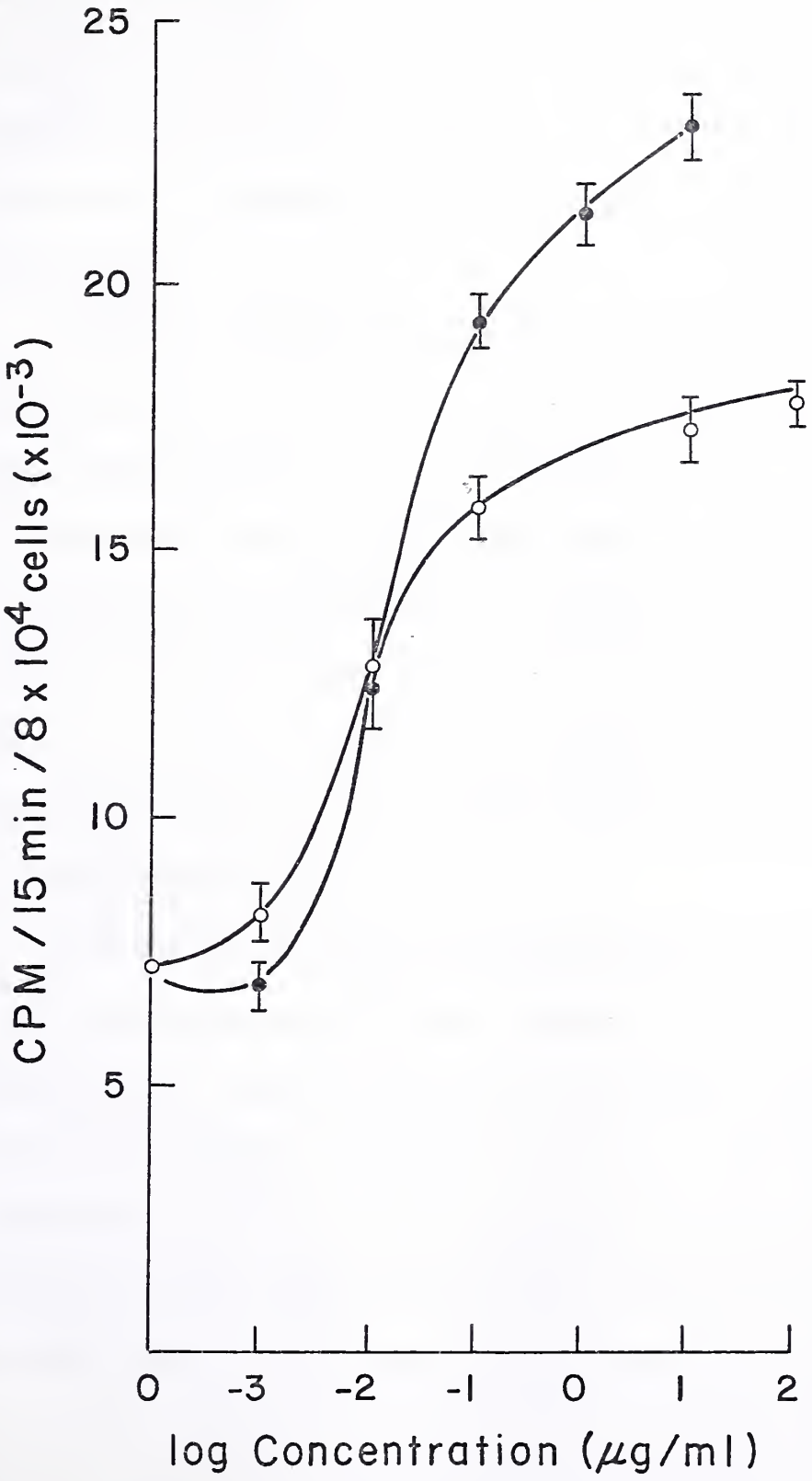
	transport (fm/15min/80,000 cells)	
	-EGF	+EGF
Thymidine	292±10	293±10
2 deoxyglucose	76±10	92±12
putrescine	348±11	696±21
spermine	450±2	720±22
α aminoisobutyric acid	9840±301	9890±113
ornithine	16940±471	16000±635

Table 2. Rate of transport of various compounds by human skin fibroblasts. In the presence and absence of EGF (1 μg/ml). All compounds were at a concentration of 10^{-7}M. Measurements were made as described in Materials and Methods after a six hour exposure to EGF.

	Putrescine transport, percent of control		
	Spermine	Spermidine	Ornithine
$-\log_{10}$ M			
3	3	5	100
4	5	5	100
5	5	4	102
6	12	11	98
7	46	55	103
8	98	87	100
9	102	98	102
10	99	95	95

Table 3. Influence of varying concentrations of spermine, spermidine, and ornithine on the transport of 10^{-7} M ^{3}H-putrescine.

FIGURE 10 . Dose response curve of the stimulation of ^{3}H-putrescine entry into human skin fibroblasts by EGF (0) and insulin (●). Measurements were made as described in Materials and Methods.


25
20
15
10
5
CPM / 15 min / 8 x 10^4 cells (x10^-3)
0
-3
-2
-1
0
1
2
log Concentration (μg/ml)

Putrescine transport is highly dependent on cell density. The transport of putrescine was inversely related to cell density (Figure 11). This relationship between putrescine transport and cell density was confirmed in fibroblasts by Pohjanpelto. The stimulation of putrescine transport by EGF was reduced at cell densities greater than 1.87×10^4 cells/cm^2 (74).

In addition to stimulating putrescine transport, EGF, insulin and FCS stimulated the proliferation of human skin fibroblasts and, to a lesser extent, KB cells (Table 4). The stimulation of cell division elicited by these three agents was significantly enhanced by putrescine and correlated with their ability to stimulate putrescine transport. ODC activity was also stimulated by EGF. The stimulation was about two fold in mouse skin and six fold in cultivated human skin fibroblasts. ODC activity in KB cells was stimulated two fold by insulin and uninfluenced by EGF (Table 5).

Cycloheximide was found to stimulate the uptake of putrescine by cultivated human skin fibroblasts, confirming the results of Pohjanpelto (74). Figure 12 shows the dose response curve of the effect of cycloheximide on putrescine transport. Concentrations of cycloheximide of 10^{-6}M or greater inhibited ^{3}H-leucine incorporation into TCA insoluble material more than 80%. The time course of stimulation shows that the effect of cycloheximide is evident between

FIGURE 11 . Effect of KB cell density on ^{3}H-putrescine transport (0) EGF (1μg/ml); (●) control. Measurements were made as described in text.

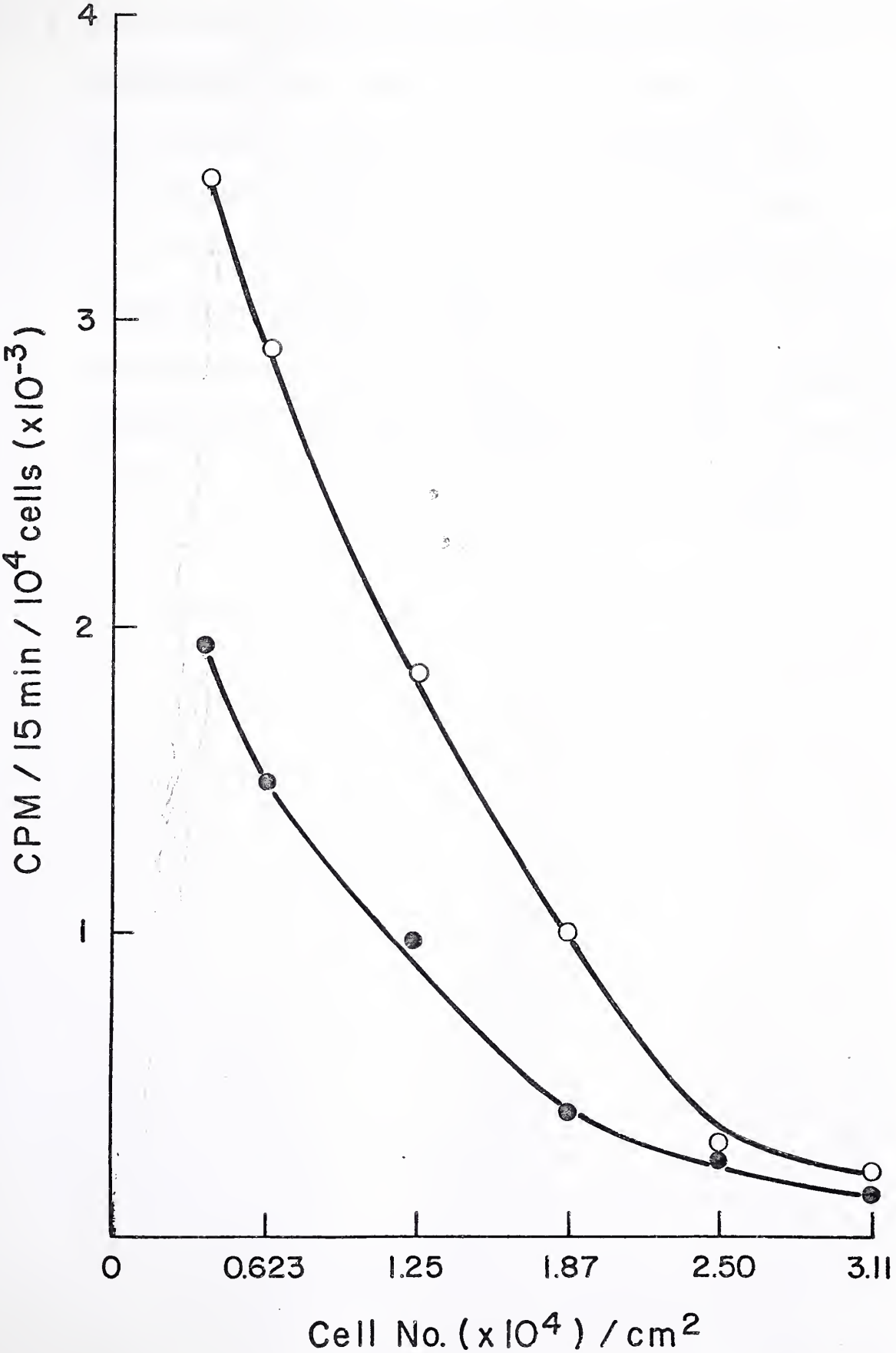

4
3
2
1
CPM / 15 min / 10^4 cells ($\times 10^{-3}$)
0
0.623
1.25
1.87
2.50
3.11
Cell No. ($\times 10^4$) / cm^2

Table 4 Effect of EGF (1 μg/ml), insulin (10 μg/ml) and FCS (10%) on proliferation of human skin fibroblasts (SF) and KB cells in the presence and absence of 2.5×10^{-8} M putrescine. Human skin fibroblasts and KB cells were seeded at 40,000 cells and 120,000 cells per 3.5 cm diameter tissue culture dish respectively in Dulbecco's medium with 10% FCS. After 24 hours, medium was removed and 2 ml of medium without serum was added. Appropriate additions in 0.2 ml of medium were made 18 hrs. later. Cells were counted 72 hours after the additions were made.

TABLE 4

Skin Fibroblasts

Treatment	without putrescine			with putrescine		
		increase from 0 time			increase from 0 time	
	cell no. ($\times 10^{-4}$)	increase in cell # $\times 10^{-4}$	% increase	cell # ($\times 10^{-4}$)	increase in cell # $\times 10^{-4}$	% increase
0	4.04±0.72	-	-	-	-	-
Control	3.94±0.11	-.06	-2.5	5.10±0.14	1.06	26
EGF	5.90±0.14	1.86	46	6.85±1.20	2.81	70
FCS	6.17±0.32	2.13	53	7.20±0.28	3.16	78
Insulin	9.27±0,10	5.23	130	10.85±0.35	6.81	168

KB Cells

Treatment	without putrescine			with putrescine		
		increase from 0 time			increase from 0 time	
	cell no. $\times 10^{-4}$	increase in cell # $\times 10^{-4}$	% increase	cell # ($\times 10^{-4}$)	increase in cell # $\times 10^{-4}$	% increase
0	1.20±0.20	-	-	-	-	-
Control	1.70±0.10	0.5	42	1.95±0.20	0.75	63
EGF	1.90±0.20	0.7	58	2.51±0.11	1.31	109
FCS	2.80±0.22	1.6	133	3.20±0.25	2.0	167
Insulin	3.40±1.5	2.2	183	3.80±0.10	2.6	217

	p Moles $^{14}CO_2$/mg protein/20 min		
	control	EGF	insulin
Mouse skin	22.3	53.8	not measured
Skin fibroblasts	8.0	49.8	80.0
KB Cells	153.0	188.0	333.0

Table 5. Effect of EGF and insulin on ODC measured according to Stastny and Cohen (32) (EGF, 1 μg/ml; insulin, 10 μg/ml).

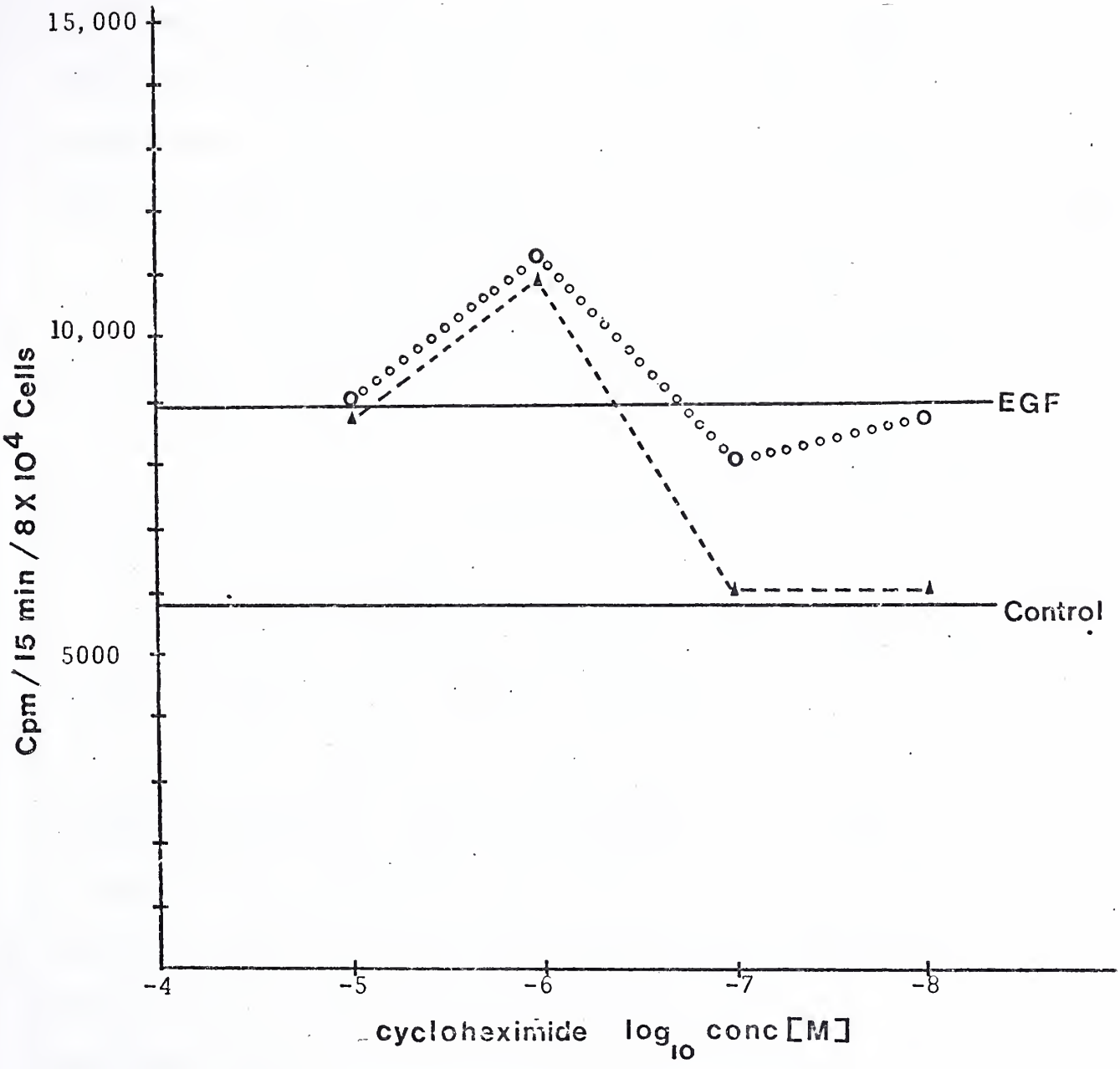


Figure 12 Dose response curve of the effect of cycloheximide on putrescine transport into human skin fibroblasts in the presence (ooooo) and absence (- - -) of EGF (1 ug/ml). Cycloheximide was added 1/2 hour before the addition of EGF. Measurements were made as described in Materials and Methods. after a six hour exposure to EGF. Values for cultures in the presence and absence of EGF without putrescine are represented by the horizontal bars.

1 and 3 hours after its addition (Figure 13). 10^{-5}M puromycin produced a similar stimulation of putrescine transport, but lower concentrations inhibited the stimulation of putrescine transport by EGF, whereas cycloheximide seemed to have little effect on the stimulation by EGF over a wide range of concentrations.

Of the group of compounds whose transport were measured, the stimulation of transport by cycloheximide was found only in the case of polyamines. Table 6 shows the comparative rates of transport of various compounds determined from the linear regions of uptake curves. It may be significant that the stimulation of transport by EGF shows a similar pattern of specificity.

The polyamines, putrescine, spermine and spermidine, are probably actively transported into the cell through the same channels. 10^{-4} fold concentrations of ornithine have no effect on the transport of either putrescine or spermine. Moreover, the addition of various amounts of spermine and/or spermidine to cultures of cells transporting ^{3}H-putrescine inhibited transport in precisely the amounts one would predict if the same channels were being used by all three polyamines (Table 7).

Curves of the velocity of putrescine transport versus concentration of putrescine indicate that the inhibition of protein synthesis by cycloheximide at 2×10^{-5}M approximately doubles the maximum velocity of transport without significantly affecting the K_m (Figure 14).

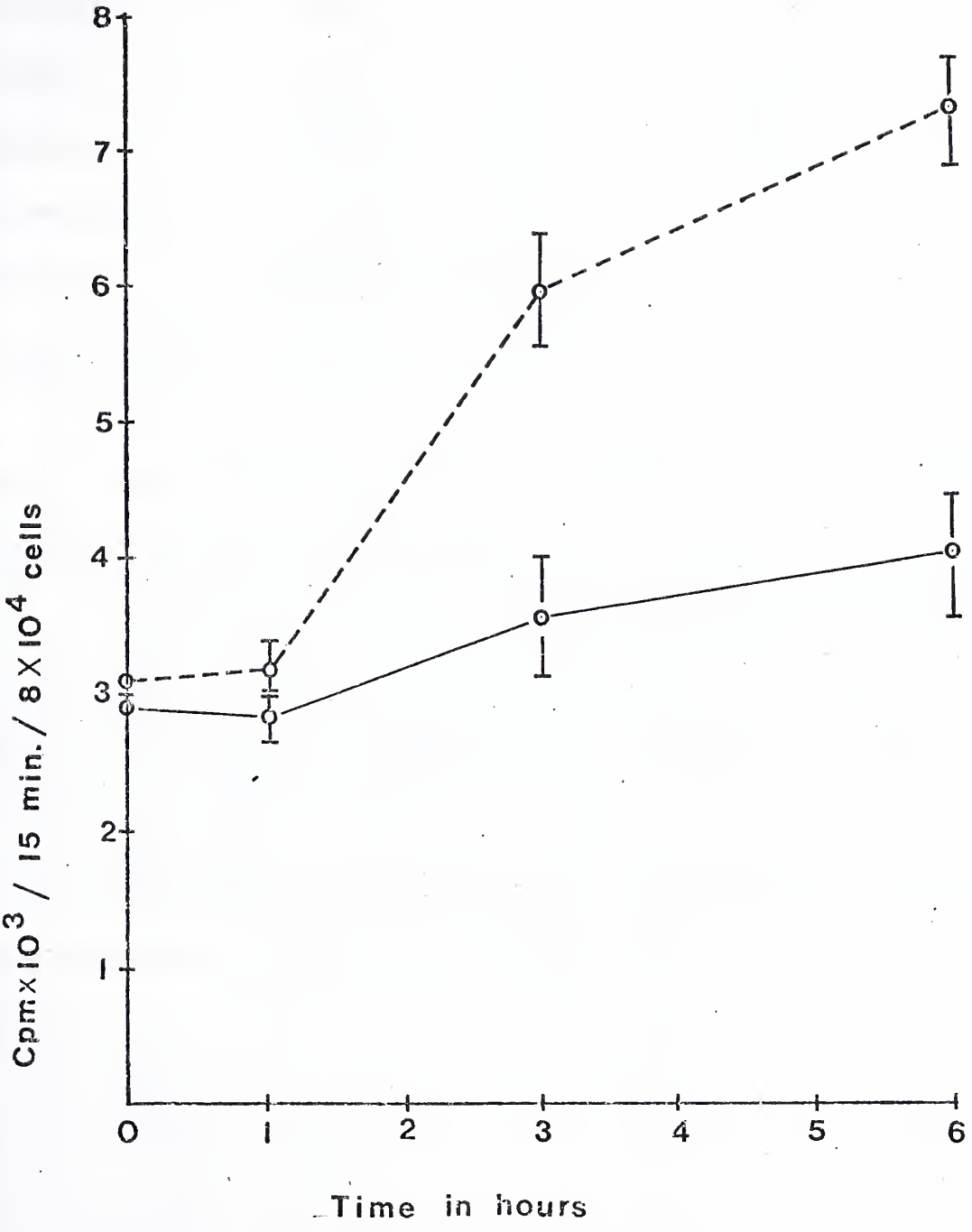


Figure 13 . Time course of the stimulation of putrescine transport by cycloheximide. Measurements were made as described in Materials and Methods. Control (———) Cycloheximide (- - - - -)

	transport (fm/15 min/80,000 cells)			
	Control	EGF	Cycloheximide	Cyclohex + EGF
putrescine	348±11	696±21	709±35	725±41
spermine	450±2	905±47	1080±127	1152±236
thymidine	292±10	293±10	190±5	204±26
2 deoxyglucose	76±10	82±12	51±5	71±5
ornithine	16940±471	16000±635		
α aminoisobutyric acid	9840±301	9890±113	5609±261	5314±452

Table 6 . Rate of transport of various compounds by human skin fibroblasts in the presence and absence of EGF (1 μg/ml) and cycloheximide($2X10^{-5}M$) All compounds were at a concentration of 10^{-7}M. Cycloheximide was added 30 minutes before the addition of EGF. Measurements were made as described in Materials and Methods after a six hour exposure to EGF. Rates were determined from linear portions of uptake curves made over a period of 20 minutes. Incorporation of ^{3}H-methyl thymidine into DNA was approximately 10% of the indicated values of uptake into the intracellular pools for all experimental cases.

polyamine concentrations (M)	rate of putrescine uptake fmoles/15 min/8×10^4 cells		% of control
		predicted	observed
putrescine (10^{-7})	360±2		-
putrescine (10^{-7}) + spermine (10^{-7})	162±5	50	45.0
putrescine (10^{-7}) + spermine (2×10^{-7})	106±10	33.3	29.5
putrescine (10^{-7}) + spermine (10^{-7}) + spermidine (10^{-7})	97±4	33.3	27.4
putrescine (10^{-7}) + spermine (2×10^{-7}) + spermidine (2×10^{-7})	66±6	25	18.4

Table 7. Effect of the addition of varying amounts of spermine and spermidine on the transport of ^{3}H putrescine. Spermine and spermidine were added 10 minutes prior to the additon of ^{3}H-putrescine. Measurements were made as described in Materials and Methods.

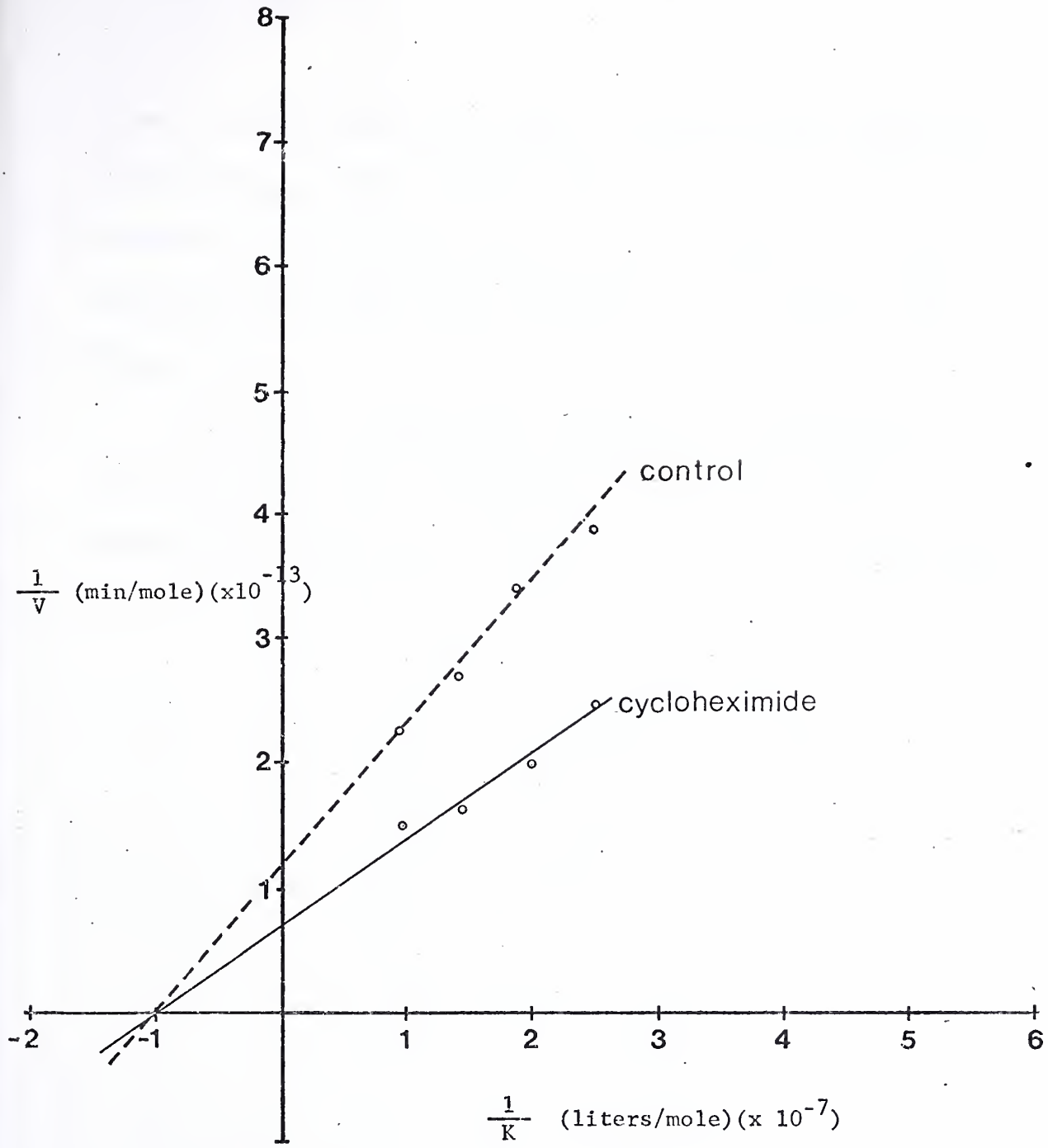


Figure 14. Michaelis-Menten plot of $\frac{1}{[\text{putrescine}]}$ vs. $\frac{1}{\text{transport velocity}}$

Control (----); cycloheximide, 2 x 10^{-5}M (———)

From the curves obtained by linear regression analysis, the calculations of V_{max} were 1.6 mmoles/minute and 3.1 mmoles/minute for untreated and cycloheximide treated cells respectively; the determinations of K_m were 2.2×10^{-7} liters/mole and 2.6 liters/mole respectively.

In contrast to cycloheximide, transport was inhibited by a variety of other agents. Cytochalasin B, colchicine, DNP and actinomycin D significantly inhibited putrescine transport (Table 8).

treatment	concentration	rate of putrescine transport % of control
control	-	100
CB	25 μg/ml	30
colchicine	10^{-7}M	67
dinitrophenol	10^{-4}M	50
	10^{-3}M	42
actinomycin D	5 μg/ml	62

Table 8 . Effect of inhibitors of mitosis, respiration, transcription, and contractility on rate of putrescine transport. Measurements were made as described in Materials & Methods. Cell viability following exposure to these compounds was demonstrated by (1) the trypan blue exclusion test of Phillips and Terryberry (76)and by (2) the resumption of cell division following removal of the drugs.

DISCUSSION

The mechanism through which EGF stimulates DNA synthesis and cell proliferation (15, 44, 47) is unknown. Our results suggest that EGF may stimulate cell proliferation by increasing intracellular levels of polyamines. We have shown that EGF increases intracellular polyamine concentration in two ways - putrescine transport and ODC activity.

Our findings suggest the existence of an active transport mechanism for the entry of polyamines into cells. Evidence for this are saturation kinetics, specificity of the substances transported, the ability of related substances to inhibit specifically the transport of putrescine, energy dependence and the unipolarity of substrate movement.

A variety of compounds tested inhibited putrescine transport. As previously reported (77) the inhibition by dinitrophenol suggests dependence on energy derived from oxidative phosphorylation. The inhibition by actinomycin D suggests that transcription is vital for the continuation of putrescine transport. The inhibition by cytochalasin B is of interest, since this compound has been reported to inhibit endocytosis (78). This could mean that putrescine is transported into the cell by endocytosis as well as by direct transport through the plasma membrane. Cytochalasin B

has also been reported though to bind to the plasma membrane (79); so a direct effect on the transport through the membrane cannot be ruled out.

Increasing cell density reduces the rate of cell division (80). This may result from a decreased ability to accumulate polyamines under these conditions (81). At high cell density, ODC activity (82) and polyamine transport (74) are both reduced as is stimulation of EGF-induced putrescine entry. All these observations suggest that putrescine (a metabolic product of ornithine) or the other polyamines (of which putrescine is a precursor) are cues for cell division.

The stimulation of putrescine or spermine transport by EGF might be coincidentally rather than casually related to growth stimulation, however the influence of EGF is specific; it stimulates only the transport of polyamines. The uptake by human skin fibroblasts of 2-DOG, thymidine, and AIB was uninfluenced by EGF. It is of particular interest that EGF did not influence the transport of ornithine, which can enter metabolic pathways other than polyamine synthesis.

Our data on the kinetics of polyamine transport show that the K_m is unaffected by EGF; however, the V_{max} of transport is increased. If a specific transport protein within the cell membrane binds to the transported substrate, carries it through the membrane, and releases it into the cell, our observations are consistent with a mechanism

whereby EGF effectively increases the number of such carrier proteins without altering their energy of binding.

Our data also show that both cycloheximide and EGF influence this particular transport mechanism specifically. Table 6 shows that cycloheximide stimulates the transport of putrescine and the related polyamine spermine but inhibits the transport of thymidine, 2-DOG, and alpha-AIB. Likewise, EGF selectively stimulates polyamine transport, but has no effect at all on the transport of the other compounds tested. This is understandable, since cycloheximide is probably affecting the synthesis of a variety of proteins that affect different transport mechanisms in different ways.

Active transport is now thought to depend on a specific transport protein within the plasma membrane to which the transported molecule binds with some degree of specificity. In light of this theory, our data suggest that a protein with a relatively short half-life (less than 3 hours) minimizes or blocks the rate of putrescine and spermine transport into the cell. This protein apparently does not affect the rate of dissociation of the substrate (putrescine) from the specific transport protein since the K_m is not altered by cycloheximide. Thus it seems likely that this protein probably affects the effective number of specific transport protein(s) and/or turnover rates rather than the molecular architecture of the transport proteins. Since cycloheximide has no immediate effect

on putrescine transport, it is more likely that its effect results from an inhibition of protein synthesis and rather than a direct binding to the plasma membrane.

The hypothesized rate-limiting protein takes on added significance with the realization that polyamines are being increasingly linked to the stimulation of cell proliferation. Diseases of growth such as neoplasms and psoriasis may be due to some extent to altered levels of such a protein.

Proteolytic enzymes appear to influence cells in a manner similar to EGF. Proteases stimulate proliferation of cultivated fibroblasts (83) and trypsin has been found to be a stimulator of ODC activity in cultivated fibroblasts (84). Trypsin has been shown to be an accelerator of putrescine transport (77) and has also been reported to stimulate cell division in a variety of cell lines when conditioned medium containing putrescine (80) was employed. These observations suggest that EGF may exert its effect upon putrescine transport by possessing an intrinsic protease activity or by stimulating a membrane protease that inactivates the transport inhibitory protein (Figure 15).

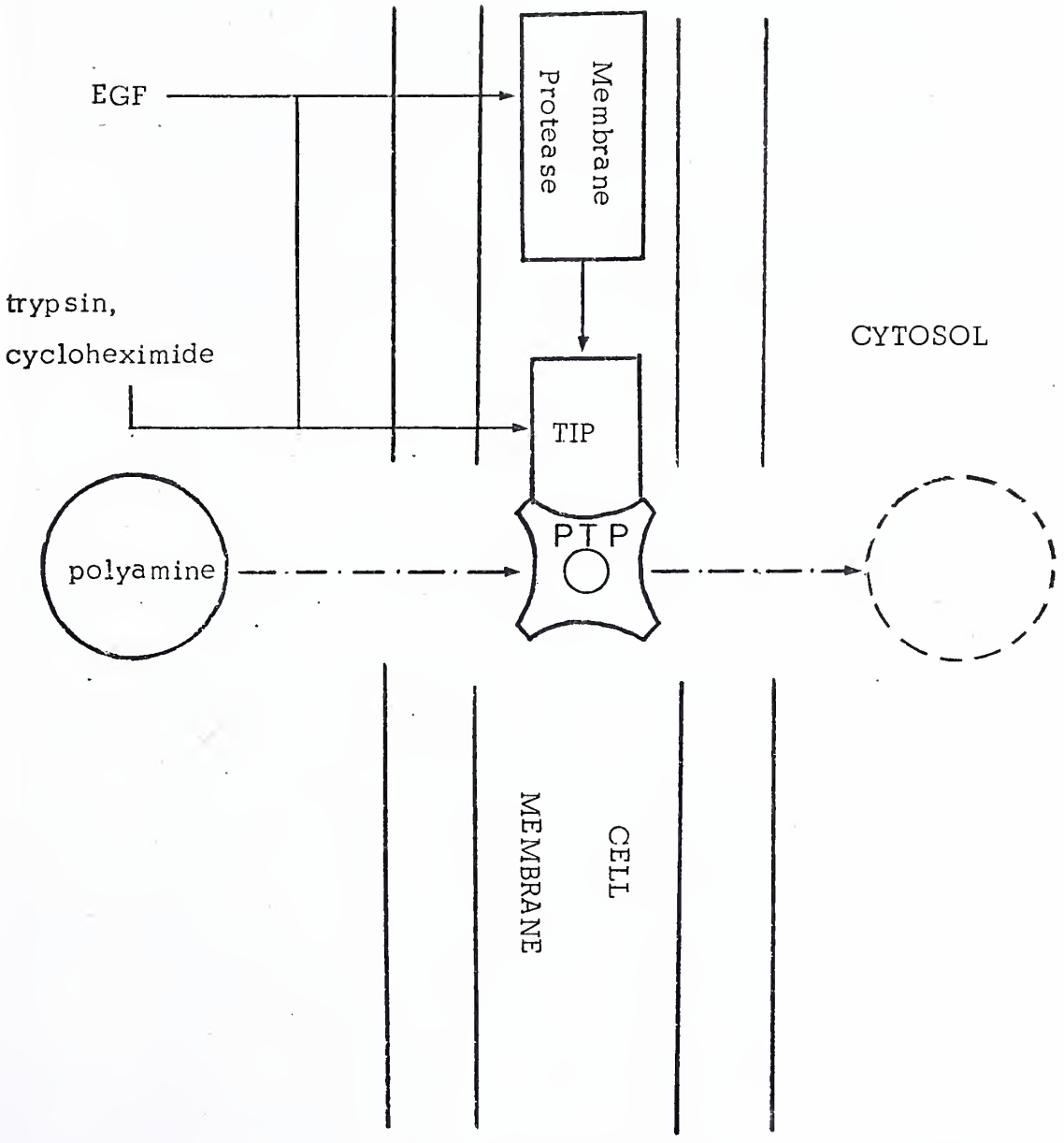


Figure 15. Schematic representation of proposed polyamine transport mechanism. EGF could bring about increased polyamine transport by affecting the inhibitory effect of transport inhibiting protein (TIP) on polyamine transport protein (PTP) via a direct, intrinsic protease activity or by stimulating a membrane associated protease.

SUMMARY

1. Epidermal Growth Factor (EGF) stimulates the rate of putrescine transport and ornithine decarboxylase activity, as well as cell proliferation, in cultivated human skin fibroblast cells.
2. This transport mechanism is specific for the polyamines.
3. A protein with a short half-life selectively inhibits this transport mechanism.
4. Inhibition of the synthesis of this inhibitory protein increases the maximum velocity of putrescine transport without affecting K_m.

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